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UNIVERSITY OF ALBERTA

Characterization of Seven Human Transfer RNA Genes

by

Domenico Spadafora

A Thesis

Submitted to the Faculty of Graduate Studies and Research

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Microbiology

Edmonton, Alberta

Fall 1993

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FACULTY OF GRADUATE STUDIES AND RESEARCH

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Dedication

To my parents,

brother and sisters

for their patience and understanding over the years

Abstract

Three human λ-Charon 4A recombinant bacteriophage clones, that had previously been shown to contain five intron-containing $tRNA^{Tyr}$ genes, were further characterized and an additional $tRNA^{Tyr}$ gene and a $tRNA^{Ala}$ gene were identified. The restriction maps of these three clones were determined using a novel partial digestion technique. The clone, λHtM4, contains a tRNA gene heterocluster consisting of two intron-containing $tRNA^{Tyr}$ genes and an alanine tRNA gene on a 2.4 kb DNA fragment. The tRNA genes on λHtM4 were all in the same orientation. Two of the clones, λHtM2 and λHtM6, were shown to overlap and the overlapping region included the sole $tRNA^{Tyr}$ gene carried by λHtM2. The four intron-containing $tRNA^{Tyr}$ genes on λHtM6, found on a 9.2 kb DNA fragment, were also all in the same orientation. The extensive homology in the flanking sequences of these genes suggests that a single progenitor $tRNA^{Tyr}$ gene gave rise to the four genes on λHtM6. The similarities in the intervening sequences are also consistent with gene duplication events. Experiments using *in vitro* transcription systems, derived from human cell lines, have shown that the $tRNA^{Tyr}$ genes and the $tRNA^{Ala}$ gene are all expressed. All of the $tRNA^{Tyr}$ genes are expressed at similar levels, except for 4-2 which does not appear to be as transcriptionally active as the others. The transcriptional efficiencies of these seven tRNA genes were not affected by deletions of their 5' and 3' flanking sequences.

The pre-tRNA transcripts obtained from *in vitro* transcription reactions have been shown to exhibit limited exhibit Mg^{2+}-dependent RNA self cleavage activity. This activity can be inhibited or modified by oligonucleotides complementary to the intron or exon sequences.

Acknowledgements

I had the privilege of working alongside very many kind and generous people during my graduate studies. Dr. Roy provided excellent supervision and set high standards both for himself and the students under his direction. He taught me very valuable lessons that will guide me in the future as I pursue a career in science. My supervisory committee; Drs. J.B. Bell, R.von Tigerstrom, and S.E. Jensen; also provided guidance and encouragement that made this work possible. With the assistance of Dr. Roy and my supervisory committee I have achieved my childhood dream of becoming a scientist.

I am very grateful to my coworkers, which include both graduate students and staff, who provided assistance as well as friendship over the years. They supplied inspiration in many forms, which included everything from brewing to bedlam. A few people that deserve special mention are Sanja Saftic´; Drs. Xiaoning Wu, Jamie Doran, Bill Henry and Tenshuk Kadima; Pat Murray; Jeff Hoyem; Donald Netolitzky and Neeraja Sankaran.

The contributions of my family are also noteworthy since without their support and encouragement I would not have made it this far. They endured my mood swings and helped me battle my depressions and obsessions.

Finally, I acknowledge the Department of Microbiology for its support, which was in the form of a teaching assistant position.

Table of Contents

1. Introduction 1

1.1 The Structure of tRNA 1

1.2 tRNA Splicing 8

1.3 RNA Polymerase III 10

1.4 *In Vitro* Transcription Systems 14

1.5 RNA Polymerase III Transcription 17

1.6 RNA Polymerase III Transcription Factors 21

1.7 Objectives of this Study 28

2. Materials and Methods 29

2.1 Materials 29

2.1.1 Chemicals and Enzymes 29

2.1.2 Recombinant Bacteriophage Clones 30

2.1.3 Bacterial Strains and Plasmids 30

2.1.4 Mammalian Cell-lines 31

2.2 Preparation of Bacteriophage DNA 31

2.3 Plasmid Isolation and Purification 32

2.4 Restriction Enzyme Digests 33

2.5 Nucleic Acid Labeling 33

2.6 Transformation and Transfection 34

2.7 Unidirectional Deletions with Exonuclease III 35

2.8 DNA Sequencing 35

2.9 Southern Cross Experiment 36

2.10 Restriction Endonuclease Mapping of Recombinant Bacteriophages λHtM2, λHtM4, and λHtM6 37

2.11 Analysis of the λHtM6 $tRNA^{Tyr}$ Gene Cluster by PCR 40

2.12 Cloning of PCR Amplified $tRNA^{Tyr}$ Genes 41

2.13 Colony Hybridization 42

2.14 Cell Extract Preparation for *in vitro* Transcription 43

2.15 *In vitro* Transcription Assays 43

2.16 RNA Synthesis *in vitro* with RNA Polymerase T7 44

2.17 Magnesium-promoted RNA Self-cleavage 45

3. Results 46

3.1 Restriction Endonuclease Mapping of λHtM2, λHtM4, and λHtM6 46

3.2 Determination of the λHtM6 $tRNA^{Tyr}$ Gene Orientations by PCR 75

3.3 DNA Sequencing 79

3.4 *In vitro* Transcription Analysis of Cloned Human tRNA Genes in Mammalian Cell Extracts 96

3.5 Self-cleavage of Pre-tRNA 133

4. Discussion 150

5. Bibliography 168

6. Appendix 189

List of Tables

		Page
Table 1	Polypeptide content of nuclear RNA polymerases from *Saccharomyces cerevisiae*	13
Table 2	Polypeptide content of RNA polymerase III from various organisms	15
Table 3	DNA fragments generated by restriction endonuclease digestion of λHtM2	63
Table 4	DNA fragments generated by restriction endonuclease digestion of λHtM6	64
Table 5	DNA fragments generated by restriction endonuclease digestion of λHtM4	76

List of Figures

		Page
Figure 1	The cloverleaf secondary structure of tRNA with the standard numbering notation	3
Figure 2	The tertiary folding patterns exhibited by tRNA	5
Figure 3	An outline of tRNA splicing	11
Figure 4	Schematic diagrams of RNA polymerase III preinitiation transcription complexes	23
Figure 5	Schematic diagram of the partial digest restriction mapping technique	38
Figure 6	Southern cross of λHtM2 against λHtM6	48
Figure 7	Southern cross of λHtM2 against λHtM4	51
Figure 8	Restriction endonuclease digestion of λHtM2, λHtM4 and λHtM6 DNAs with *Hin*dIII	53
Figure 9	Comparison of λHtM2 and λHtM6 restriction fragments carrying $tRNA^{Tyr}$ genes	56
Figure 10	Identification of λHtM2 restriction fragments carrying a $tRNA^{Tyr}$ gene	58
Figure 11	Identification of λHtM6 restriction fragments carrying specific $tRNA^{Tyr}$ genes	60
Figure 12	Restriction endonuclease mapping of λHtM4 by partial digestion	66
Figure 13	Identification of λHtM4 restriction fragments carrying $tRNA^{Tyr}$ genes	68
Figure 14	Selection of λHtM4 restriction fragments carrying $tRNA^{Tyr}$ genes for subcloning	71
Figure 15	Identification of pJM4 restriction fragments carrying $tRNA^{Tyr}$ genes	73

Figure 16 Restriction endonuclease digestion maps of the three recombinant bacteriophage DNAs 77

Figure 17 Determination of the λHtM6 tRNA^{Tyr} gene orientations by PCR
Panel A 80
Panel B 82

Figure 18 Schematic representation of the PCR products extending from gene to gene on recombinant bacteriophage λHtM6 84

Figure 19 Nucleotide sequence of the tRNA^{Tyr} gene within pM6128 86

Figure 20 Nucleotide sequence of the tRNA genes within pJM4 89

Figure 21 Nucleotide sequence of the tRNA^{Tyr} gene within pM6 93

Figure 22 Nucleotide sequence of the tRNA^{Tyr} gene within pM612 97

Figure 23 Nucleotide sequence of the tRNA^{Tyr} gene within pM6IT-E 101

Figure 24 Nucleotide sequence alignment of the λHtM6 tRNA^{Tyr} genes and their flanking sequences 104

Figure 25 Nucleotide sequences of the tRNA^{Tyr} genes cloned into pBS 108

Figure 26 Restriction endonuclease maps of the recombinant plasmids containing the tRNA genes from λHtM4 110

Figure 27 Restriction endonuclease maps of recombinant plasmids containing the 6-1 tRNA^{Tyr} gene 112

Figure 28 Restriction endonuclease maps of recombinant plasmids containing the 6-2 tRNA^{Tyr} gene 114

Figure 29 Restriction endonuclease maps of the recombinant plasmids containing the 6-3 tRNA^{Tyr} gene 116

Figure 30 Restriction endonuclease maps of the recombinant plasmids containing the 6-4 tRNA^{Tyr} gene 118

Figure 31 RNA transcripts from *in vitro* transcription reactions directed by recombinant plasmids containing cloned human tRNA genes 121

Figure 32 RNA transcripts from *in vitro* transcription reactions directed by recombinant plasmids containing the 6-1 tRNA^{Tyr} gene 123

Figure 33 RNA transcripts from *in vitro* transcription reactions directed by recombinant plasmids containing the 6-2 $tRNA^{Tyr}$ gene 125

Figure 34 RNA transcripts from *in vitro* transcription reactions directed by recombinant plasmids containing the 6-3 $tRNA^{Tyr}$ gene 127

Figure 35 RNA transcripts from *in vitro* transcription reactions directed by recombinant plasmids containing the 6-4 $tRNA^{Tyr}$ gene 129

Figure 36 RNA transcripts from *in vitro* transcription reactions directed by recombinant plasmids containing tRNA genes from λHtM4 131

Figure 37 Autoradiograph of the 4-1 pre-$tRNA^{Tyr}$ transcripts and their cleavage products 136

Figure 38 Autoradiograph of the 4-1, 6-2 and 6-3 pre-$tRNA^{Tyr}$ transcripts and their cleavage products 138

Figure 39 Autoradiograph of the 4-1, 6-4 and 6-1 pre-$tRNA^{Tyr}$ transcripts and their cleavage products 140

Figure 40 Autoradiograph of the 6-1 and 6-4 pre-$tRNA^{Tyr}$ transcripts and their cleavage products 142

Figure 41 Autoradiograph of the 6-3 and 6-2 pre-$tRNA^{Tyr}$ transcripts and their cleavage products 144

Figure 42 Autoradiograph of the 4-1 pre-$tRNA^{Tyr}$ and the 4-3 pre-$tRNA^{Ala}$ transcripts and their cleavage products 146

Figure 43 Comparison of the 4-3 $tRNA^{Ala}$ gene sequence with $tRNA^{Ala}$ gene sequences from the literature 152

Figure 44 Comparison of the isolated $tRNA^{Tyr}$ gene sequences with $tRNA^{Tyr}$ gene sequences form the literature 155

List of Abbreviations

amp	ampicillin
bp	base pair(s)
cos	cohesive
ICR	internal control region
IPTG	isopropyl-β-thiogalactoside
kb	kilobase pairs(s) (or 1000 base pairs)
NaOAc	sodium acetate
nt	nucleotide(s)
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
pre-tRNA	precursor tRNA
Ψ	pseuoduridine
SDS	sodium dodecyl sulfate
snRNA	small nuclear RNA
SSPE	standard saline phosphate EDTA
TAFS	TATA-binding protein-associated factors
TBP	TATA-binding protein
TE	10 mM Tris-HCl, 1 mM EDTA pH 8.0
TFIIIA	transcription factor IIIA
TFIIIB	transcription factor IIIB
TFIIIC	transcription factor IIIC
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Y	pyrimidine
R	purine

1. Introduction

1.1 The Structure of tRNA

The principal function of transfer RNA (tRNA) is to serve as an adapter in the translation of a nucleotide sequence in messenger RNA into the amino acid sequence of a protein, which involves interaction of the tRNA with both the ribosome and the mRNA. All functional tRNAs are able to occupy the P and A sites of the ribosome, to allow the mRNA and the tRNA anticodon to pair as the polypeptide chain is elongated. In order for tRNAs to associate with translation factors it is necessary for them to share certain characteristics. Their adaptor function, however, requires that they also be distinguishable. This strict requirement is especially evident when tRNA aminoacylation occurs. The aminoacyl-tRNA synthetase brings together the amino acid, an appropriate tRNA and ATP in order to synthesize an aminoacyl-tRNA. As an example of how subtle a determinant for aminoacylation can be, the G3:U70 base pair (bp) in the acceptor stem of *Escherichia coli* $tRNA^{Ala}$ is the major feature allowing the cognate aminoacyl-tRNA synthetase to identify this tRNA (Hou and Schimmel, 1988; Francklyn *et al.*, 1992). The tRNAs aminoacylated by a single aminoacyl-tRNA synthetase are referred to as isoaccepting tRNAs.

A compilation of tRNA and tDNA sequences, prokaryotic and eukaryotic, has shown that some positions are invariant (i.e., present in >90-95% of tRNAs). The invariant positions include U_8, A_{14}, G_{18}, G_{19}, A_{21}, U_{33}, G_{53}, T_{54}, Ψ_{55}, C_{56}, A_{58}, C_{61}, C_{74}, C_{75}, and A_{76} (Sharp et al., 1985). There are also semi-invariant positions that are occupied by either a pyrimidine (Y) or a purine (R); for example, Y_{11}, R_{15}, R_{24}, Y_{32}, R_{37}, Y_{48}, R_{57}, and Y_{60} (Sprinzl *et al.*, 1989). The secondary structure features of tRNAs can be summarized by a universal cloverleaf structure. All tRNAs conform to this general secondary structure (with the exception of some mitochondrial tRNAs which exhibit a modified form of it) by having sequences which allow base pairing between short

complementary regions. The cloverleaf secondary structure has four common features which include the acceptor arm, the TΨC arm, the D arm, and the anticodon arm (Figure 1). The acceptor arm consists of the 5' and 3' ends of the molecule. The 3' end contains an unpaired sequence to which the amino acid is esterified at either the 2' or 3' hydroxyl group. Another feature of the cloverleaf structure is the extra arm, the most variable of the secondary features, that divides tRNAs into two classes. These two classes are distinguishable by the size of the extra arm, with Class 1 tRNAs having an arm of only 3-5 bases and Class 2 tRNAs having an arm of 13-21 bases.

The first three-dimensional tRNA structure elucidated was that of yeast $tRNA^{Phe}$; it was determined to have an "L-shaped" backbone by X-ray crystallography (Kim *et al.* 1974, Figure 2). This tertiary structure suggested that all tRNAs are likely to adopt the "L-shape" as a result of tertiary hydrogen bonds and that most of the conserved and semi-conserved bases are involved in tertiary hydrogen bonding (Kim 1978).

The presence of a variety of modified bases is a characteristic feature of tRNAs. More than fifty modifications have been identified in tRNAs (reviewed in Bjork and Kohli, 1990). In most cases these modifications arise from enzymatic modification of an existing base. These modifications are not restricted to the bases; methylation at the 2'-O position of the ribose also occurs. An instance where the modification is not to an existing base is the formation of Q bases, where a tRNA transglycosylase exchanges free queuosine for a guanosine residue in the tRNA (Okada *et al.*, 1979).

The numbers of tRNA genes present in the genomes of several organisms, both prokaryotic and eukaryotic, have been estimated using the technique of RNA-DNA hybridization. Hatlen and Attardi (1971) estimated 1300 tRNA sites per human haploid genome, based on saturation and competition hybridization experiments using a purified, radioactively labeled tRNA fraction. By further fractionating isolated tRNA using reverse-phase chromatography, it was concluded that there are more than 80 isoaccepting tRNA species encoded in the human genome (Lin and Agris, 1980). To obtain numbers for a

Figure 1. The cloverleaf secondary structure of tRNA with the standard numbering notation.

The invariant bases are indicated by the actual base, while the semi-invariant bases are shown either as Py for pyrimidine or Pu for purine. An asterisk (*) indicates bases that are usually modified in the mature tRNA. The small circled numbers represent nucleotides which are not present in all tRNAs. The terminal CCA, shown as shaded circles, is always added post-transcriptionally to eukaryotic tRNAs but is encoded in some prokaryotic tRNA genes. The arrow indicates the intron splice site. This figure has been adapted from Kim *et al.* (1974).

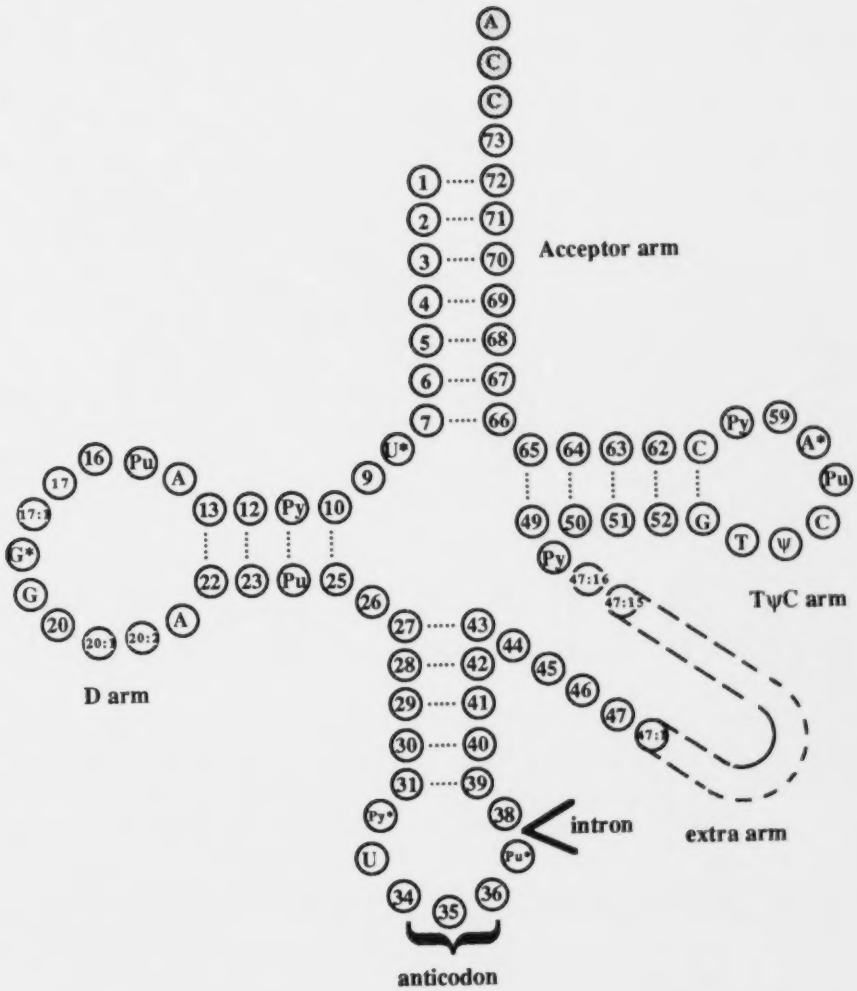
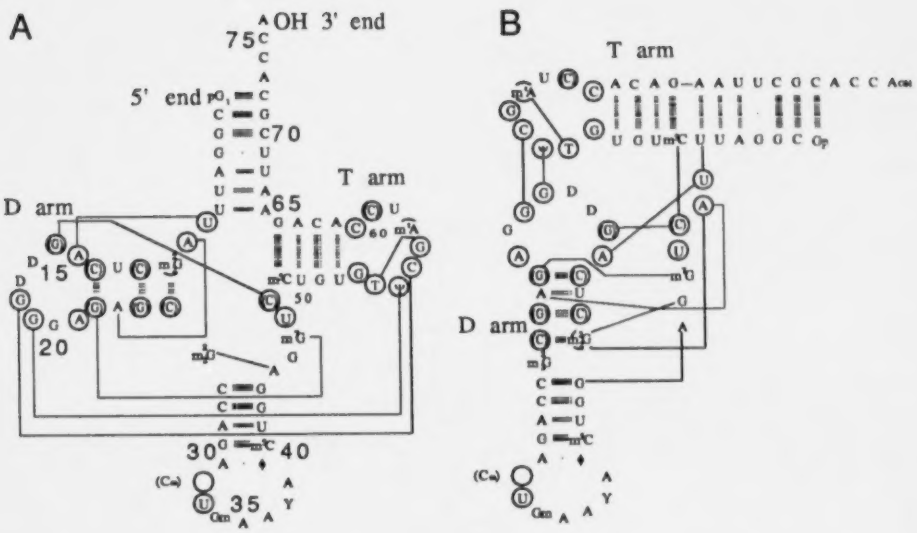
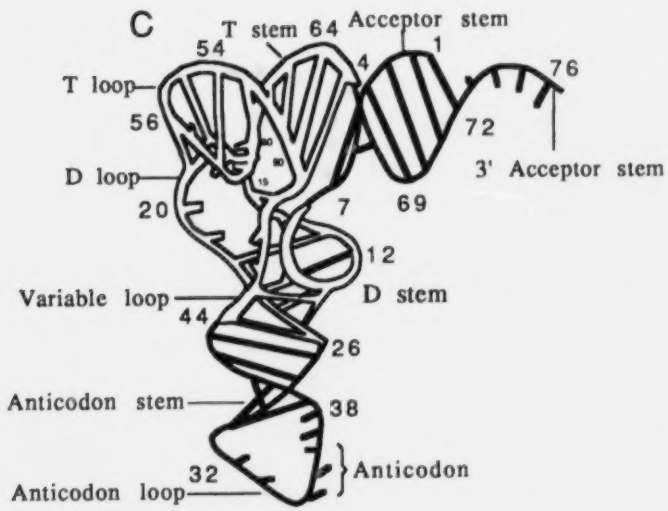

A
C
C
73
1
72
2
71
3
70
4
69
5
68
6
67
7
66
Acceptor arm
U*
9
16
Pu
A
17
17:1
G*
G
20
20:1
20:2
A
13
12
Py
10
22
23
Pu
25
26
D arm
65
64
63
62
C
Py
59
A*
Pu
49
50
51
52
G
T
ψ
C
Py
47:16
47:15
TψC arm
27
43
44
28
42
45
29
41
46
30
40
47
47:1
31
39
Py*
38
intron
extra arm
U
Pu*
34
35
36
anticodon

Figure 2. The tertiary folding patterns exhibited by tRNA.

The bases that appear unpaired in the cloverleaf structure (panel A) are usually involved in base pairing with other regions of the tRNA molecule to assist in achieving the final L- shaped tertiary structure as shown in the yeast $tRNA^{Phe}$ example (panel C). The folding of the D and T arms in the tRNA tertiary structure is stabilized by hydrogen bonds and base stacking interactions. This figure was adapted from Kim *et al.* (1974).


A
OH 3' end
5' end
T arm
D arm
B
T arm
D arm


C
T stem
Acceptor stem
T loop
D loop
3' Acceptor stem
Variable loop
D stem
Anticodon stem
Anticodon loop
Anticodon

particular tRNA species, total genomic DNA restriction endonuclease digests have been probed with either highly purified tRNA species or DNA fragments encoding tRNA genes. This strategy was used to detect 13 $tRNA^{Val}$ (Arnold *et al.*, 1986), 12 $tRNA_i^{Met}$ (Santos and Zasloff, 1981), and 12 $tRNA^{Tyr}$ (van Tol and Beier, 1988) gene-containing fragments. These values are at best estimates of the actual numbers of the respective tRNA species since it is quite possible for a restriction fragment to harbor more than one gene and it is also possible for the probe to hybridize with pseudogenes. The term pseudogene is used to refer to DNA sequences that consist of either a partial tRNA gene or a tRNA gene-like structure for which no novel RNA species has been or, perhaps, can be isolated (Sharp *et al.*, 1985).

In the human genome, tRNA genes occur either in clusters, both homo- and hetero-clusters, or as individual genes. A few human tRNA gene clusters have been cloned and characterized. Roy *et al.* (1982) cloned and sequenced a 1.65 kb fragment of DNA from a human-λ recombinant that contained $tRNA^{Lys}$, $tRNA^{Gln}$, and $tRNA^{Leu}$ genes separated from one another by about 0.5 kb. A homocluster of four $tRNA^{Tyr}$ genes was described by MacPherson and Roy (1986), which was also the first example of human tRNA genes with intervening sequences. Shortridge *et al.* (1989) reported a 6 kb fragment from a human-λ recombinant containing a heterocluster consisting of $tRNA^{Thr}$, $tRNA^{Pro}$, and $tRNA^{Val}$ genes. Chang *et al.* (1986) have characterized a human-λ recombinant which has four tRNA genes (two $tRNA^{Pro}$, $tRNA^{Thr}$, and $tRNA^{Leu}$) on a 8.2 kb *Hin*dIII fragment. Doran *et al.* (1987) have described a tRNA cluster consisting of two $tRNA^{Phe}$ and two $tRNA^{Lys}$ genes. Examples of individually occurring human tRNA genes include $tRNA^{Gly}$ (Shortridge *et al.*, 1985; Pirtle *et al.*, 1986), $tRNA^{Glu}$ (Goddard *et al.*, 1983), and $tRNA^{Asn}$ (Ma *et al.*, 1984). Originally one of the $tRNA^{Gly}$ genes described by Doran *et al.* (1988) was thought to be a solitary gene; however, it has since been shown by Morrison *et al.* (1991) to be linked to the tRNA gene cluster previously described by Roy *et al.* (1982). As more gene mapping, cloning and sequencing projects

are undertaken other tRNA genes, once considered isolated, may become linked to known tRNA gene clusters.

1.2 tRNA Splicing

While intron-containing tRNA genes are common in *Saccharomyces cerevisiae*, which has ten intron-containing isoaccepting tRNA gene families (Ogden *et al.*, 1984; Stucka and Feldmann, 1988), they are uncommon in higher eukaryotes. However, tRNA splicing is essential in all eukaryotes since all known $tRNA^{Tyr}$ genes contain introns (MacPherson and Roy, 1986; van Tol and Beier, 1988). Introns in precursor tRNAs (pre-tRNAs) do not have any consensus sequences, even at the splice junctions, however they are always located one nucleotide to the 3' side of the anticodon and do not alter the mature tRNA domain (Szekely *et al.*, 1988). The splicing of a pre-tRNA consists of an endonucleolytic excision of the intron, by a specific endoribonuclease, with subsequent ligation of the 5' and 3' halves to form the mature tRNA sequence (Peebles *et al.*, 1983).

It has been shown that the pre-tRNAs have a common tertiary structure, with the tRNA portion adopting the L-shaped conformation, the 3' splice site always being single-stranded, and the intron probably on the surface of the molecule available to the splicing endoribonuclease (Lee and Knapp, 1985). It is these common secondary and tertiary structural features the enzyme must recognize since a single endoribonuclease can cleave all intron-containing pre-tRNAs (Peebles *et al.*, 1983). By studying the effects of nucleotide substitutions in pre-tRNAs of both *Xenopus* and yeast it has been determined that the splice sites are chosen according to the length of the anticodon stem (Greer *et al.*, 1987; Mattoccia *et al.*, 1988; Reyes and Abelson, 1988). The splicing endoribonuclease (the endonuclease which excises introns) cleaves the pre-tRNA generating a 5' half-molecule with a terminal 2', 3'-cyclic phosphate and a 3' half-molecule beginning with a 5' hydroxyl group (Peebles *et al.*, 1983). In yeast this endoribonuclease is an integral membrane protein composed of three subunits (Rauhut *et al.*, 1990), while in *Xenopus* the

endoribonuclease is soluble (Gandini-Attardi *et al.*, 1985). Similar endoribonucleases have been found in HeLa cells (Laski *et al.*, 1983) and in wheat germ (Stange *et al.*, 1988). In addition to the biochemical evidence, in yeast there is also genetic evidence that this endoribonuclease is involved with tRNA splicing since temperature-sensitive mutants accumulate pre-tRNA splicing intermediates (Ho et al., 1990; DeMarini *et al.*, 1992). There has also been a report of non-enzymatic pre-tRNA intron excision from human $tRNA^{Tyr}$ precursors (van Tol *et al.*, 1989). However, no other reports supporting this claim have appeared. In that paper, van Tol *et al.* (1989) proposed that the role of the splicing endoribonuclease was to assist the pre-tRNA in attaining the proper conformation for autocatalytic intron excision and the prevention of non-specific self-cleavage.

Two types of ligases have been found which join tRNA half-molecules. One of the best characterized of these is the yeast tRNA ligase, which is a 95-kD protein (Westaway *et al.*, 1988). This enzyme possesses three distinct catalytic activities required for ligation: a cyclic phosphosdiesterase to open the cyclic phosphate, a kinase to phosphorylate the 3' half-molecule, and an adenylase which ligates the half-molecules (Phizicky *et al.*, 1986; Apostol *et al.*, 1991). When the yeast tRNA ligase joins the 5' and 3' half-molecules the resulting mature sequence tRNA bears a 2'-phosphate at the splice junction. The removal of the 2'-phosphate is carried out by a NAD-dependent 2'-phosphate-specific dephosphorylating enzyme, which has been observed *in vitro* in HeLa extracts (Zillmann *et al.*, 1991) and in yeast extracts (McCraith and Phizicky, 1990, 1991). This type of ligase has also been detected in wheat germ (Konarska *et al.*, 1981). The other type of ligase ligates the 5' and 3' half-molecules without the resulting 2'-phosphate (Nishikura and De Robertis, 1981). This type of ligase was first characterized in HeLa cell extracts, where it was found to be approximately 160 kD and capable of ligating RNAs bearing 5' hydroxyl and 2',3' cyclic phosphate termini in an ATP-requiring reaction (Filipowicz *et al.*, 1983; Perkins *et al.*, 1985). Although this type of ligase is the principal ligase in vertebrates, a yeast tRNA ligase-like activity has also been found in HeLa cells, suggesting that both the

endonuclease and tRNA ligase reactions are evolutionarily conserved in eukaryotes (Zillmann *et al.*, 1991). These two types of ligase are distinguishable by determining the origin of the junction phosphate. The yeast-like tRNA ligase incorporates a phosphate from ATP into the splice junction, while the HeLa-like tRNA ligase uses the phosphate derived from the 5' precursor backbone as the junction phosphate (Nishikura and De Robertis, 1981; Figure 3).

1.3 RNA Polymerase III

There are three types of eukaryotic nuclear RNA polymerases. This classification was originally based on the separation of three forms of RNA polymerase by DEAE-Sephadex chromatography. Each form has been further characterized according to its chromatographic properties, ionic strength optimum, divalent cation requirement, template preference and α-amanitin sensitivity. While these criteria are sufficient to differentiate RNA polymerases of a particular cell type, the specific characteristics of each polymerase are not necessarily universal among eukaryotes. The eukaryotic RNA polymerases have been designated RNA polymerase I, II, and III. They are also known as RNA polymerase A, B, and C, respectively. Each RNA polymerase transcribes its own set of genes; RNA polymerase I transcribes ribosomal RNA precursors, RNA polymerase II transcribes messenger RNA precursors, as well as some small nuclear RNAs (snRNAs), and RNA polymerase III transcribes 5S rRNA, tRNAs, some snRNAs and several small viral RNAs.

Analysis of purified eukaryotic RNA polymerases has shown each to be a multi-subunit enzyme composed of two large distinct polypeptides and of several smaller polypeptides, with some of the polypeptides common to all three forms (Sentenac, 1985; refer to Table 1). The *Saccharomyces cerevisiae* nuclear RNA polymerases have been studied extensively, with the genes for several subunits having been cloned. There are three subunits that appear to be shared among the *S. cerevisiae* nuclear RNA polymerases. They have molecular masses of 27, 23, and 14.5 kD and are encoded by the RPB5, RPB6,

Figure 3. An outline of tRNA splicing.

The first step in tRNA splicing is the excision of the intervening sequence by an endoribonuclease, which generates 2', 3'-cyclic phosphate and 5' hydroxyl ends. The subsequent ligation of the exons can be carried out by two specific types of tRNA ligases: a HeLa-like tRNA ligase and a yeast-like tRNA ligase. The HeLa-like tRNA ligase joins the 5' and 3' half-molecules by a direct reaction between the 2', 3'-cyclic phosphate and 5' hydroxyl ends. The yeast-like tRNA ligase prepares the half-molecules for ligation by hydrolysis of the 2', 3'-cyclic phosphate to yield a 2' phosphate with a free 3'-hydroxyl, phosphorylation of the 5'-hydroxyl group, and the adenylation of the 5' phosphate. The adenylation reaction is indicated on the diagram as the addition of A-P- to the 5' phosphate of exon 2. The ligation reaction leaves a 2' phosphate at the splice junction which is removed by a NAD-dependent 2' phosphate-specific phosphatase. Once the 2' phosphate is removed, a 5' - 3' phosphate linkage is left at the splice junction. Portions of this figure were adapted from Phizicky *et al.* (1992) and from Zillmann *et al.* (1991).

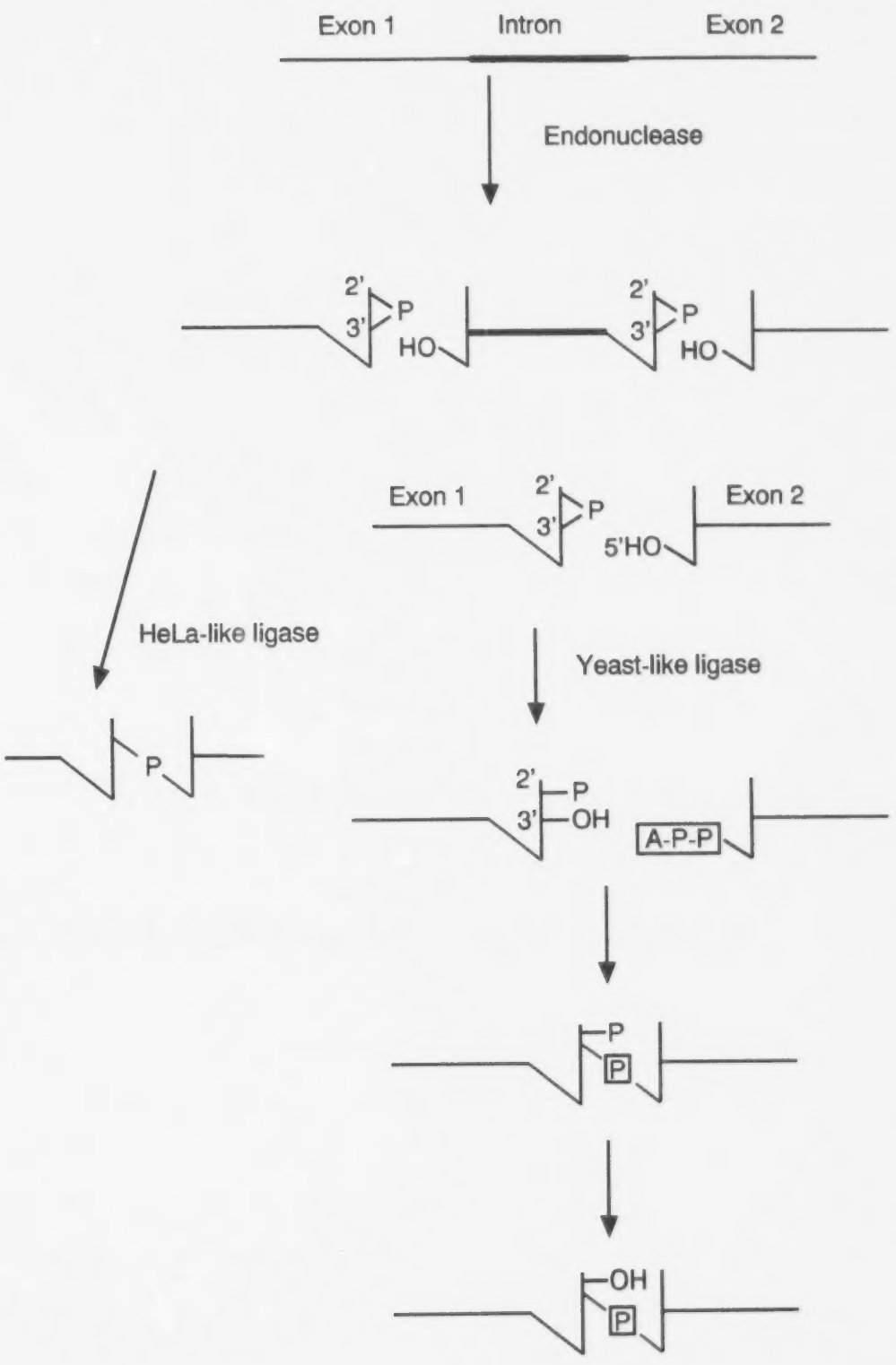

Exon 1
Intron
Exon 2
Endonuclease
2'
3'
P
HO
2'
3'
P
HO
Exon 1
2'
3'
P
5'HO
Exon 2
HeLa-like ligase
Yeast-like ligase
P
2'
3'
P
OH
A-P-P
P
P
OH
P

Table 1. Polypeptide content of the nuclear RNA polymerases from *Saccharomyces cereivisae*[a]

Pol I	Pol II	Pol III
190	220	160
135	150	128
49	44.5	82
43	32	53
40	27*	40
34.5	23*	37
27*[b]	16	34
23*	14.5*	31
19	12.6	27*
14.5*		23*
14		19
12.2		14.5*
10		11
		10

[a] Polypeptides are identified by their molecular weights in kD. The molecular weights cited for the subunits were obtained from Huet *et al.* (1985) and from Sentenac (1985).

[b] Common polypeptides are identified with an asterisk (*).

and RPB8 genes, respectively (Woychik *et al.*, 1990). The product of the RPB6 gene has been detected in all three RNA polymerases by immunoprecipitation, suggesting that this common subunit is identical in all three RNA polymerasaes (Woychik *et al.*, 1990). Human RNA polymerase III was first purified from KB cells by Roeder and coworkers (Jaehning *et al.*, 1977). The RNA polymerase III subunits range in size from less than 10 kD to approximately 170 kD, as determined by SDS polyacrylamide gel electrophoresis (Table 2). The molecular weight of RNA polymerase III varies from 600-680 kD in the characterized enzymes as determined by analysis of their subunit components, assuming that each subunit is represented once.

1.4 *In Vitro* Transcription Systems

Transcription by RNA polymerases II and III has been investigated using *in vitro* techniques, as had been done with the bacterial RNA polymerase. Initially, *in vitro* transcription systems were based on isolated *Xenopus laevis* oocytes (Ng *et al.*, 1979) or nuclei (Birkenmeier *et al.*, 1978; Schmidt *et al.*, 1978) made from these oocytes which transcribed genes from chromatin. *In vitro* systems that contain isolated nuclei are of limited value for studying transcription because manipulation of the active components is difficult and transcription of exogenous genes requires the injection of DNA templates into the nuclei. As limited as these early systems were, they still provided insight into the basic requirements for eukaryotic gene transcription. It was observed that chromatin isolated from immature *Xenopus laevis* oocytes contained endogenous RNA polymerase activity that synthesized predominantly 5S rRNA. Supplementing the RNA polymerase activity with exogenous purified RNA polymerase III stimulated total RNA and 5S rRNA synthesis up to 50 fold (Parker and Roeder, 1977). However, oocyte RNA polymerase has been shown to transcribe cloned 5S rRNA genes on recombinant plasmids in a near random fashion. These studies suggested that chromatin-associated proteins are required for the selective and asymmetric transcription of the 5S rRNA genes in *Xenopus laevis*

Table 2. Polypeptide content of RNA polymerase III from various organisms[a]

Saccharomyces cerevisiae[b]	*Acanthamoeba castellanii*[c]	*Podospora comata*[d]	Wheat germe[e]	*Bombyx mori*[f]	*Drosophila hydei*[g]	*Xenopus laevis*[h]	Mouse plasmacytoma[i]	HeLa cells[j]
160	169	174	150	155	154	155	155	155
128	138	129	130	136	135	138	138	138
82	82	87	94	67	62	94	89	86
53	52	50	55	62	58	68	70	63
40	39-37	39	38	49	38	41	53	43
37	34	23	30	39	32	39	49	34
34	30	21	28	36	31	33	41	32
31	28.5	19	25	31	27.2	29	33-32	27
27	22.5	17	24.5	28	26.5	24	29	22
23	17.5	16.5	20.5	18	21.5	23	19	
19	15.5	13.5	20		17.5	19		
14.5	13.3	11	19.5					
11	<10	10	17.8					
			17					

[a] Polypeptides are identified by their molecular weights in kD.
[b] Huet *et al.* (1985)
[c] D'Alessio *et al.* (1979)
[d] Barreau and Begueret (1982)
[e] Jendrisak (1981)
[f] Sklar *et al.* (1976)
[g] Gundelfinger *et al.* (1980)
[h] Engelke *et al.* (1983)
[i] Sklar and Roeder (1976)
[j] Jaehning *et al.* (1977)

oocytes. Similar observations were made using a human transcription system consisting of isolated nuclei from KB cells (Jaehning and Roeder, 1977). Both the *Xenopus laevis* and the human KB cell expression systems indicated very strongly that additional factors were required for selective RNA polymerase III transcription. A modification of these expression systems did allow the expression of endogenous and exogenous genes; however, it was a laborious technique which involved microinjecting template DNA into *Xenopus laevis* oocytes (Kressmann *et al.*, 1978). The first DNA-dependent, soluble transcription system was reported by Wu (1978). This transcription system used exogenous DNA, ribonucleoside triphosphates and a cell-free post-mitochondrial supernatant (S-20) from human KB cells. This methodology took advantage of the fact that most of the RNA polymerase III leaches out of the nucleus when cells are lysed hypotonically, which allows nuclei, mitochondria, ribosomes and other cell debris to be removed by centrifugation. The supernatant also contains, in addition to RNA polymerases, other factors necessary for accurate transcription. Using this soluble transcription system, Wu demonstrated that the VA_1 gene from purified adenovirus 2 DNA was selectively transcribed by RNA polymerase III, based on transcription experiments which included α-amanitin. Weil *et al.* (1979) also described a similar DNA-dependent, soluble transcription system that is derived from a high speed centrifugation (S-100) of a cytoplasmic fraction from cultured cells, based on the method of Wu and Zubay (1974). Another transcription system was described by Manley *et al.* (1980) which was initially described as a HeLa cell RNA polymerase II system, but has since been shown to have considerable RNA polymerase III activity. This transcription system consists of a lysate containing RNA polymerase and transcription factors obtained from HeLa cell nuclei. Initially the proteins are stripped from the chromatin by ammonium sulphate, allowing the DNA and cell debris to be removed by centrifugation. The remaining supernatant is further treated with ammonium sulphate to concentrate its transcription activity. Soon after these transcription systems were described, several

similar protocols were developed for other eukaryotic cell lines and organisms, and, aside from some minor modifications, these expression systems are still in use for studying gene expression and regulation. Some examples of soluble, DNA-dependent transcription systems include *Bombyx mori* silkgland extracts (Sprague *et al.*, 1980), nematode extracts (Honda *et al.* 1986), *Drosophila* cell extracts (Dingermann *et al.*, 1981; Rajput *et al.*, 1982) and yeast extracts (Klekamp and Weil, 1982).

1.5 RNA Polymerase III Transcription

Transcription studies of RNA polymerase III genes revealed an unexpected result in 1980, when it was discovered that these genes have an internal promoter (Sakonju *et al.*, 1980; Bogenhagen *et al.*, 1980). The first RNA polymerase III-dependent promoter determined was for a *Xenopus* somatic 5S rRNA gene. Through a series of deletions which removed 5' and 3' flanking and coding sequences, it was determined that base pairs 50 to 83 were absolutely required for accurate initiation of transcription (Sakonju *et al.*, 1980; Bogenhagen *et al.*, 1980). Studies of tRNA gene transcription suggested that these genes are controlled by two regions, one of which is external and another which is internal (DeFranco *et al.*, 1980; Kressmann *et al.*, 1979; Sprague, *et al.*, 1980). The internal control region is responsible for a basal level of transcription and the external control region in some cases acts to modulate the transcription from the internal promoter. This model for a tRNA gene promoter was further refined in 1981 with the discovery of the split internal promoter of a tRNA gene (Galli *et al.*, 1981; Hofstetter *et al.*, 1981; Sharp *et al.*, 1981). The tRNA gene promoter was characterized by deletions of the 5' and 3' flanking sequences until transcription was abolished. The same strategy identified the 5S rRNA gene internal promoter. Sharp *et al.* (1981) studied the transcription of several modified versions of a *Drosophila* $tRNA^{Arg}$ gene construct using a variety of homologous and non-homologous *in vitro* transcription systems. The deletion studies suggested that the first $tRNA^{Arg}$ transcription control region resided between nucleotides 8 and 25 and

the second between nucleotides 50 and 58, based on the numbering of the mature tRNA sequence. A similar study of transcription of the *Xenopus laevis* $tRNA^{Arg}$ gene by Hofstetter *et al.* (1981) mapped its internal control regions, the first between nucleotides 8 to 13 and the second between nucleotides 51 to 72. Galli *et al.* (1981) mapped the split internal promoter sequence of a *Xenopus laevis* $tRNA^{Leu}$ gene to nucleotides 13 through 20 and nucleotides 51 through 64 and they also coined the terms A block and B block to describe these internal regions, respectively. The analogous promoter elements in the 5S rRNA gene are referred to as the A and C boxes (Geiduschek and Tocchini-Valentini, 1988).

The A block is contained within the sequence that codes for the D loop and has also been termed the D-control region, A box, or 5' internal control region (ICR). The B block is contained within the sequence that codes for the T loop and has also been termed the T- control region, B box, or 3' ICR (Sharp *et al.*, 1985). These initial reports suggested that the internal split promoter elements are common to all eukaryotic tRNAs, since the A and B blocks closely coincide with two conserved sequence blocks that are present in all eukaryotic tRNA genes. These regions are conserved due to the presence of invariant nucleotides, of which box A contains U_8, A_{14}, G_{18}, and G_{19}, while box B contains G_{53}, T_{55}, C_{56}, A_{58}, and C_{61}. These conserved regions were thought to be important solely from the point of tRNA structure and function. It is now apparent that these sequences are also important as gene promoters. This point became evident when *E. coli* $tRNA^{Asp}$ and $tRNA^{Trp}$, which have strong A and B homologies, were shown to yield specific transcripts in a eukaryotic transcription system while *E. coli* $tRNA^{Tyr}$, which has weak A and B homologies, was transcriptionally inactive (Galli *et al.*, 1981; Melton and Cortese, 1979). These findings supported the observations made by Koski *et al.*, (1980) who studied point mutations of a yeast $tRNA^{Tyr}$ gene (SUP4) and found that invariant nucleotides are important for gene expression. The tRNA gene promoter sequences were investigated further by Ciliberto *et al.* (1982), who studied the transcription of hybrid

tRNA genes constructed from existing genes of *Caenorhabditis elegans*. These experiments showed that hybrid genes are efficiently transcribed regardless of the overall structure of the tRNA genes, proving that A and B blocks are independent transcriptional signals. They also constructed mutants of the *C. elegans* $tRNA^{Pro}$ gene which had variable spacing between the promoter regions and observed residual transcription when separated by as much as 140 nucleotides. However, optimal transcription occurred when the two regions were separated by approximately 40 to 50 nucleotides. It has been established that the B box is the major determinant of promoter strength. If the A box is deleted substitutes which determine a new start point for transcription are found readily (Johnson *et al.*, 1984; Wilson *et al.*, 1985). Transcription experiments such as these and others, using tRNA gene constructs carrying internal deletions and substitutions, have defined box A and box B as having the approximate coordinates of nucleotides 8-19 and nucleotides 52-62, respectively. By aligning the promoter sequences (noncoding strand) of several RNA polymerase III-dependent genes, Galli *et al.* (1981) proposed consensus sequences for the 5' ICR as TGGCNNAGTGG and for the 3' ICR as GGTTCGANNCC. By aligning the promoter sequences of only eukaryotic tRNA genes, Sharp *et al.* (1985) determined the 5' ICR consensus as GTGGCNNAGT..GGT.AGNGC and the 3' consensus as GGTTCGANTCC. A comparison of these consensus sequences suggests that there are more constraints on the 3' ICR than on the 5' ICR.

The class III genes which contain ICRs have been divided into two sets, type 1 and type 2, with 5S rRNA genes as the only member of the type 1 set. Type 1 ICRs contain the A and C boxes, while type 2 ICRs (found in tRNA and viral-associated genes) contain the A and B boxes (Geiduschek and Tocchini-Valentini, 1988; Kunkel, 1991).

Ever since tRNA genes were first expressed *in vitro*, reports suggesting that extragenic sequences influence transcription have appeared in the literature (refer to the Appendix for a summary). The most common observation is that deletions of the 5' flanking sequence, usually within 20 bp of the transcription start site, reduce transcription

in vitro. These *in vitro* results have been supported by *in vivo* experiments with suppressor tRNA genes, which also demonstrated that deletions of the 5' flanking sequence reduced the expression of these genes. However, deletion of the 5' flanking sequence is not always detrimental to expression. In fact these deletions occasionally cause an increase in transcriptional activity (Hipskind and Clarkson, 1983). Although highly conserved sequences which act as universal extragenic control elements have not been found, there are examples of specific sequences that modulate the expression of particular tRNA genes.

While the promoters of most genes transcribed by RNA polymerase III are internal, there is another set of genes transcribed by RNA polymerase III that contain promoter elements in the 5' flanking regions. No significant amount of intragenic sequence is required for their transcription, either *in vitro* or *in vivo*. The vertebrate U6 snRNA genes and a human 7SK RNA gene are members of this class of RNA polymerase III genes (reviewed by Kunkel, 1991). Transcription of vertebrate U6 snRNA genes, unlike the invertebrate U6 genes, is dependent on three rather than two upstream elements. These three upstream elements include a TATA-like sequence, the proximal sequence element, and the distal sequence element, composed mainly of the octamer motif, ATGCAAAT, which is involved in the transcription of the 7SK RNA gene (Murphy *et al.*, 1987). Increased U6 snRNA gene transcription by RNA polymerase III was observed when additional TATA box binding protien (TBP: see Section 1.6) was introduced into *in vitro* transcription reactions (Margottin *et al.*, 1991). The TATA-like sequence element determines the polymerase specificity of the U6 gene, since the removal of the element leaves a functional RNA polymerase II-type snRNA promoter and the addition of this element to a RNA polymerase II-type snRNA promoter creates a RNA polymerase III external promoter (Mattaj *et al.*,1988; Lobo and Hernandez, 1989).

1.6 RNA Polymerase III Transcription Factors

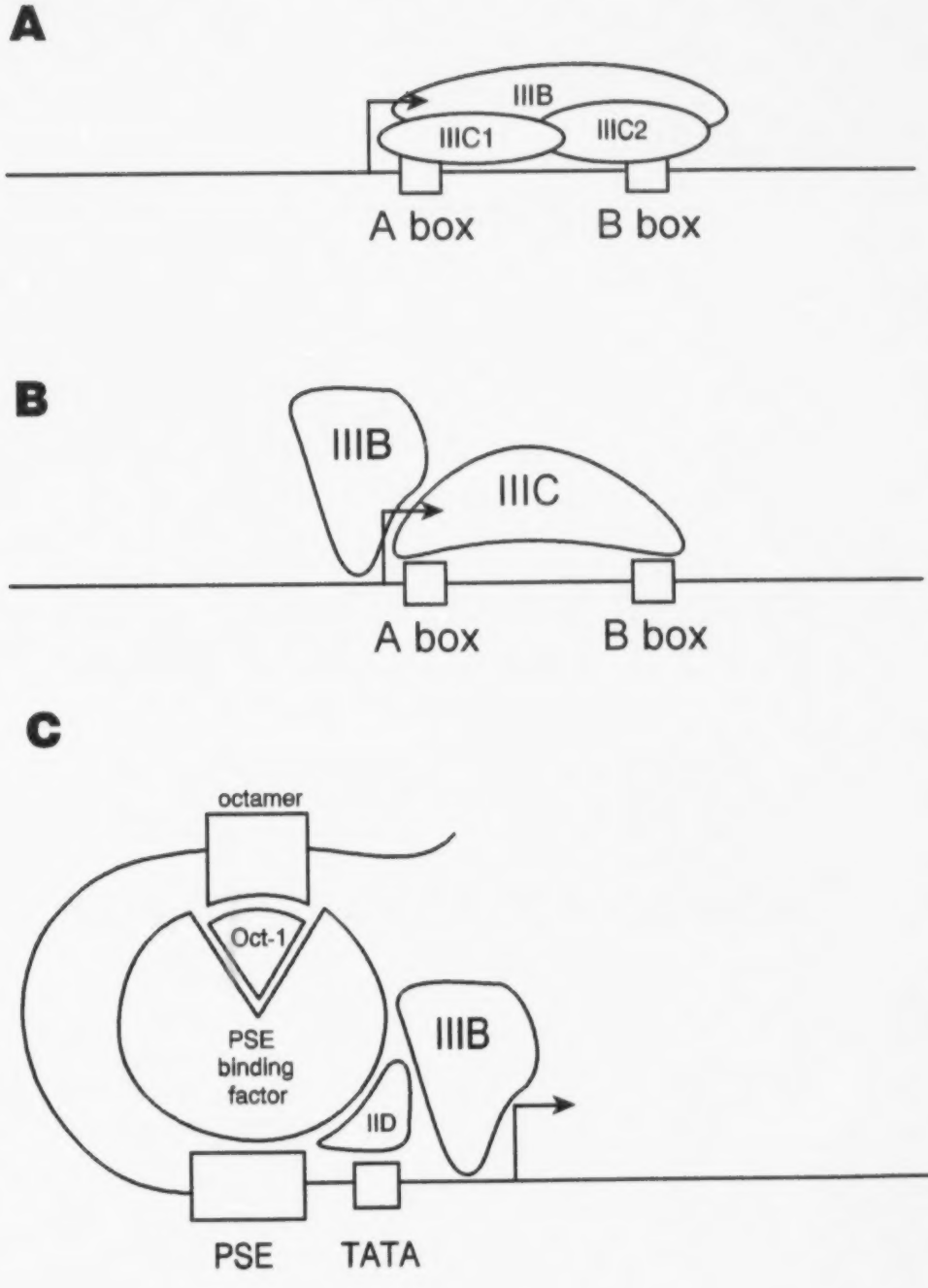
As soon as soluble, DNA-dependent transcription systems were described, work began on characterizing the functional components that are responsible for transcription. Segal *et al.* (1980) fractionated cell-free extracts (S-100) of mammalian KB cells, using a phosphocellulose column, into four different fractions containing transcription factors necessary for transcription by RNA polymerases II and III. An alternative to traditional chromatographic methods for transcription factor purification and characterization has been biological fractionation by centrifugation, which selectively sediments transcription complexes. This method takes advantage of the specific binding of transcription factors to template molecules which forms complexes stable enough to allow quantitative and selective sedimentation from an *in vitro* transcription system (Culotta *et al.*, 1985; Jahn *et al.*, 1987). Lassar *et al.* (1983), using a two-step incubation-competition assay, determined that the formation of stable pre-initiation complexes on specific templates precluded transcription of competing templates added subsequently. For the accurate transcription of a *X. laevis* $tRNA_i^{Met}$ gene and an adenovirus 2 VA_1 gene by RNA polymerase III, transcription factors TFIIIB and TFIIIC were required. In contrast, accurate transcription of a *X. borealis* 5S rRNA gene by RNA polymerase III required transcription factors IIIA, IIIB, and IIIC (Lassar *et al.*, 1983). Baker and Hall (1984) fractionated a yeast RNA polymerase III transcription system and also found two fractions (B and C) which were required for the transcription of yeast tRNA genes. The yeast $tRNA^{Arg}$ and $tRNA^{Ser}$ genes were able to form stable pre-initiation complexes with fraction C alone, while the $tRNA_3^{Leu}$ and $tRNA^{Tyr}$ genes formed stable complexes only when both fractions C and B were present. The $tRNA_3^{Leu}$ gene could be made to form stable complexes with fraction C alone by reducing the A to B block distance from 74 nucleotides to between 34 and 53 nucleotides. However, this approach did not permit $tRNA^{Tyr}$ to form stable complexes with fraction C alone. Therefore, it was concluded that

the distance between the internal control regions and sequence changes in either the A or B block affect complex stability. Alteration of the A and B block sequences towards the consensus sequence increases complex stability while alteration of these sequences away from the consensus sequence decreases complex stability (Baker and Hall, 1984). Template competition assays were conducted under conditions in which each necessary component was made limiting to define the order with which the transcription factors interact with a tRNA gene (Dean and Berk, 1988). These observations of Dean and Berk (1988) were the basis of their model for the formation of stable transcription complexes (Figure 4).

Human TFIIIC can be resolved into two components, TFIIIC1 and TFIIIC2, that bind to the A and B boxes respectively (Dean and Berk, 1987; Yoshinaga *et al.*, 1987). In a human system it was established that the first step in complex formation is the binding of TFIIIC2 with the tRNA gene B block. The next step is the binding of either TFIIIB or TFIIIC1 with the tDNA-TFIIIC2 complex since, regardless of which factor binds, the transcription of a competitor template is precluded. HeLa cell extracts made from adenovirus-infected cells transcribe tRNA and adenovirus VA genes at more than 10-fold higher levels than HeLa cell extracts made from uninfected cells (Berk, 1986; Yoshinaga *et al.*, 1986). It was found that RNA polymerase III transcription is stimulated by a product of the E1A gene, which is found in the chromatographic fraction containing TFIIIC (Hoeffler and Roeder, 1985). Enhanced RNA polymerase II and RNA polymerase III transcription *in vitro* can be achieved by adding baculovirus-produced recombinant E1A protein to a soluble DNA-dependent transcription system (Patel and Jones, 1990). The phenomenon of transcriptional activation of promoters for class II and class III genes has been observed not only with E1A protein but also with hepatitis B virus X-gene product and SV 40 t antigen (Aufiero and Schneider, 1990). Adenovirus E1A enhances RNA polymerase III-dependent transcription by promoting the phosphorylation of TFIIIC. The changes in TFIIIC phosphorylation were observed by gel mobility shift assays that

Figure 4. Schematic diagrams of RNA polymerase III preinitiation transcription complexes.

One of the most accepted models for the RNA polymerase III preinitiation transcription complex was proposed by Dean and Berk (1988) (panel A). This model has evolved as more and more experiments were performed. Based on the data from Bartholomew *et al.* (1991) the model can now be drawn with TFIIIB interacting less with TFIIIC and covering more upstream sequence (panel B). While the preinitiation transcription complex on tRNA gene templates does not incorporate upstream regulatory elements, the preinitiation transcription complex on vertebrate U6 snRNA genes (Lobo *et al.*, 1991) relies exclusively on upstream regulatory elements (panel C). The arrows indicate the start sites of transcription.


A
IIIB
IIIC1
IIIC2
A box
B box
B
IIIB
IIIC
A box
B box
C
octamer
Oct-1
PSE
binding
factor
IIIB
IID
PSE
TATA

revealed two distinct TFIIIC-promoter complexes, which suggested two forms of TFIIIC (Hoeffler *et al.*, 1988). However, adenovirus E1A does not cause an increase in TFIIIC expression (Green *et al.* 1988).

In yeast the equivalent of TFIIIC is a multisubunit factor named tau (τ), consisting of two large DNA-binding domains, τ_A and τ_B, of about 300 kD each. Each domain protects about 30 bp of DNA, as determined by several footprinting experiments using DNase I, λ exonuclease and dimethylsulfate. This was observed regardless of both the spacing between the A and B blocks and the relative helical orientation of these blocks (Baker *et al.*, 1987; Camier *et al.*, 1990). The τ_B domain has been isolated after partial proteolysis and it retains its B block binding ability, while no such τ_A-tDNA complex has yet been detected (Marzouki *et al.*, 1986). Examination by scanning transmission electron microscopy of τ and τ-tDNA complexes shows two globular protein domains, with each domain binding to a promoter element (Schultz *et al.*, 1989). Instances of DNA looping were observed by scanning transmission electron microscopy when complexes formed between τ and tRNA genes with elongated spaces (82-99) between the A and B blocks. With the wild type $tRNA_3^{Leu}$ gene these two domains are clearly separated on the DNA molecule, suggesting an apparent dissociation reaction upon binding to the A and B blocks. However, the electron microscopy data cannot exclude the existence of a hinge region connecting the τ_A and τ_B domains which would also accommodate differences in A and B block spacing and the variety of relative helical orientations of these blocks (Schultz *et al.*, 1989). From the data collected about τ and the τ-tDNA interaction it appears that the flexibility of this interaction, with regard to either the distance between or the relative helical orientation of the promoter element, is due largely to the τ protein itself (Camier *et al.*, 1990). By incorporating 5-[N-(p-azidobenzoyl)-3-aminoallyl]-deoxyuridine triphosphate, a photoreactive nucleotide analog, into specific sites within the *S. cerevisiae* SUP4 $tRNA^{Tyr}$ gene, four of five τ/TFIIIC-associated polypeptide chains have been crosslinked to this gene. The association of these polypeptides with the yeast SUP4 gene

was judged specific by the lack of crosslinking to extraneous sites and the ability of these polypeptides to compete for their respective binding sites. From a compilation of experimental results, Bartholomew *et al.* (1990) were able to determine that the 145 kD subunit is accessible to crosslinking from the vicinity of the B box, the 95 and 55 kD subunits are located on opposite sides of the helix in the vicinity of the A box, and the 135 kD subunit is crosslinked to a region of the A box and to the sequence between the A and B boxes. Their results compare favourably to an earlier report by Gabrielsen *et al.* (1989) that found four polypeptides of 145, 135, 100, and 65 kD specifically associated with a tRNA gene.

On tRNA gene templates the prior binding of TFIIIC to the intragenic promoter is required before TFIIIB can be bound, thereby resulting in a highly stable transcription preinitiation complex which is resistant to dissociation by either high ionic strength or heparin. Footprinting experiments have shown that the addition of TFIIIB to a TFIIIC-tDNA complex protects approximately 45 bp of upstream sequence, but enhances the digestion of 3-5 bp immediately upstream of the transcription start site by DNase I (Kassavetis *et al.*, 1989). Transcription factor TFIIIB has been highly purified from yeast cells (Klekamp and Weil, 1987) and from HeLa cells (Waldschmidt *et al.*, 1988), and based on SDS-PAGE, was thought to consist primarily of a 60 kD protein. However, only a small fraction (< 1%) of the yeast 60 kD protein in the purified TFIIIB fraction was capable of incorporation into a transcription complex (Kassavetis *et al.*, 1989).

Reports of a transcription stimulating factor, which bound sequences upstream of 5S rRNA and tRNA genes and was responsible for the protection of 5' flanking sequences from DNase I digestion, suggested that the properties once thought to be associated with TFIIIB were due instead to another factor. This new factor(s) could offer an explanation for the transcription modulation effect of 5' flanking sequences observed in 5S rRNA and tRNA genes (Kassavetis *et al.*, 1990; Oei and Pieler, 1990). The confusion about TFIIIB properties was resolved by photocrosslinking experiments, using 5-[N-(p-azidobenzoyl)-3-

aminoallyl]-deoxyuridine triphosphate, which provided evidence that two polypeptides (70 kD and 90 kD) have the properties of TFIIIB and that these polypeptides are separate and distinct components of yeast TFIIIB (Bartholomew *et al.*, 1991). The 70 kD polypeptide assembles onto TFIIIC-tDNA complexes, while the 90 kD polypeptide can bind to the complex only after the 70 kD polypeptide has bound, and once both polypeptides are bound the complex becomes resistant to disassociation by heparin and generates the typical DNase I protection pattern of TFIIIB. The earlier reports which described the major component of TFIIIB as a 60 kD protein were most likely due to a degradation product of either the 70 kD or 90 kD polypeptide, in light of the low complex binding activity of the 60 kD protein (Kassavetis *et al.*, 1991). Further evidence in support of the 70 kD polypeptide as being part of TFIIIB comes from genetic experiments with *Saccharomyces cerevisiae*, where mutants with reduced RNA polymerase III transcriptional activity have been isolated that lack a functional 70 kD TFIIIB subunit (Buratowski and Zhou, 1992; Colbert and Hahn, 1992).

TBP, once thought to be restricted to only RNA polymerase II promoters, has been shown to be a necessary component for transcription by all three nuclear RNA polymerases (Cormack and Struhl, 1992; Schultz *et al.*, 1992). Recent reports have shown that transcription of class III genes can be significantly inhibited by sequestering TBP from cell extracts with TATA element-containing oligonucleotides, prior to the addition of DNA template and nucleoside triphosphates (White *et al.*, 1992). The genes used in these experiments ($tRNA^{Gln}$, $tRNA^{Leu}$, 5S rRNA, VA_1, B1, and B2 genes) lack TATA boxes; however, the results indicate that TBP is involved in RNA polymerase III transcription. Further experiments have shown that the inhibitory effects of TBP depletion, either by sequestration on TATA element-containing oligonucleotides or by heat inactivation, on RNA polymerase III transcription can be alleviated by the addition of cloned human TBP, expressed in *E. coli* (White *et al.*, 1992).

The assembly of the TBP and the TATA-binding protein-associated factors (TAFS) into complexes generates the TFIID transcription factor, which is specific for RNA polymerase II promoters (Sharp, 1992). In mammalian cells, 10 polypeptides that range in size from 10-200 kD have been identified as TAFS (Pugh and Tjian, 1991). It is quite possible that the RNA polymerase specificity is determined by the subset of TAFS that combine with TBP in the complex; however, this model remains only hypothetical (Sharp, 1992).

1.7 Objectives of this Study

The main objective of this study was to identify and to sequence all of the human tRNA genes (as well as their flanking sequences) carried by three λ-human recombinant bacteriophages (λHtM2, λHtM4, and λHtM6).

A second objective was to compare the effect of varying their 5' flanking sequence on their rates of transcription with mammalian cell extracts. It was hoped that any differences observed in the *in vitro* expression of these genes (six intron-containing $tRNA^{Tyr}$ genes and one $tRNA^{Ala}$ gene) could be correlated with the presence or absence of extragenic sequences, which modulate the *in vitro* expression of these genes. The localization of regulatory elements was attempted by changing various tRNA genes with modified flanking sequences and expressing these constructs *in vitro*. The ultimate goal of this study was to attribute differences in tRNA gene transcription rates to modifications made to the native flanking sequences.

A third objective was to investigate the previously reported self-excision of intervening sequence from pre-$tRNA^{Tyr}$ transcripts.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and Enzymes

Cell culture supplies, including minimum essential medium powder, trypsin, penicillin G, streptomycin sulfate, and fetal bovine serum, were purchased from Gibco BRL. All nucleotides, including 2'-deoxyadenosine-5'-O-(1-thiotriphosphate) were purchased from Pharmacia in a lyophilized form and subsequently reconstituted as 10 mM stock solutions. The radioisotopically labeled nucleotides, [α-^{32}P]-dATP and [γ-^{32}P]-ATP, were purchased either from New England Nuclear or ICN Biochemicals Inc. Nitrocellulose and nylon transfer membranes were purchased from Amersham. Autoradiography was performed using either Kodak XAR5 X-ray film or Fuji XR X-ray film, supplied by Innomed Imaging. Agarose gels were made using low electroendosmosis agaroses from Boehringer Mannheim. Polyacrylamide gels were made using acrylamide from either Boehringer Mannheim or Bethesda Research Laboratories, and N,N'-methylene bisacrylamide from BDH. All oligonucleotides used in this study were synthesized by the Department of Microbiology DNA Synthesis Facility, University of Alberta, using Applied Biosystems model 381A or 391EP DNA synthesizers. Polymerase chain reaction (PCR) amplifications were performed using a Techne PHC-2 thermocycler. DNA quantification was performed using a Hoeffer TKO 100 mini-fluorimeter, based on the binding of Hoechst 33258 dye specifically to DNA.

All restriction enzymes were purchased either from Boehringer Mannheim, New England Biolabs, Pharmacia, or Bethesda Research Laboratories. T4 DNA ligase was purchased either from Boehringer Mannheim or Bethesda Research Laboratories. T4 polynucleotide kinase was purchased from Pharmacia. Taq DNA polymerase was purchased from Boehringer Mannheim. The Klenow fragment of *E. coli* DNA

polymerase I was purchased either from Boehringer Mannheim or Bethesda Research Laboratories.

2.1.2 Recombinant bacteriophage clones

The recombinant bacteriophages, λHtM2, λHtM4, and λHtM6, characterized in this study, were originally isolated by MacPherson (1988). The *E. coli* strain, LE 392, used to propagate the recombinant bacteriophages, was a gift from Dr. C. Strobeck, Department of Zoology, University of Alberta.

2.1.3 Bacterial strains and plasmids

Plasmid pBS (formerly pBluescribe) was obtained from Stratagene, while *E. coli* strains MV 1193 and MV 1183, M13 phage M13KO7, and plasmids pUC118 and pUC119 were gifts from J. Vieira, formerly of the Department of Biochemistry, University of Minnesota, USA. These *E. coli* strains, and M13KO7 helper phage, were propagated using the growth conditions described by Yanisch-Perron *et al.* (1985) and Vieira and Messing (1987). The $tRNA^{Tyr}$ gene-containing recombinant plasmids (pM6, pM6IT, pM6128, pM612, and pJM4) were constructed by MacPherson (1988) from the recombinant bacteriophages λHtM4 and λHtM6 using pAT153 (Twigg and Sherratt, 1980). Variations of these $tRNA^{Tyr}$ gene subclones were constructed by deleting varying amounts of 5' and/or 3' flanking sequences from each of the initial $tRNA^{Tyr}$ gene plasmid subclones. For purposes of identification, the $tRNA^{Tyr}$ genes carried on the recombinant plasmids pM6128, pM6, pM612 and pM6IT are named 6-1, 6-2, 6-3 and 6-4 respectively. The subclone of λHtM4, pJM4, contains two $tRNA^{Tyr}$ genes and one $tRNA^{Ala}$ gene. The $tRNA^{Tyr}$ gene characterized by MacPherson (1988) was named 4-1, while the second $tRNA^{Tyr}$ gene and the $tRNA^{Ala}$ gene were named 4-2 and 4-3, respectively.

2.1.4 Mammalian cell-lines

The two mammalian cell lines used in this study were HeLa cells (WT/ED/M5) and 293 cells. The HeLa cells (WT/ED/M5) were a gift from Dr. A. R. P. Paterson, MacEachern Cancer Research Laboratory, University of Alberta. The 293 cells (Graham *et al.*, 1977) were a gift from Dr. Arnold J. Berk, Molecular Biology Institute, Department of Microbiology, University of California, Los Angeles.

2.2 Preparation of bacteriophage DNA

Recombinant bacteriophages λHtM2, λHtM4, and λHtM6 were propagated as described by Maniatis *et al.* (1982). Cells were pelleted from an overnight 50 mL culture of *E. coli* LE 392 by centrifugation at 3000 × g for 10 minutes and resuspended in sterile SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$ and 0.01% gelatin) yielding a suspension that contained 10^{10} cells per mL. Two 2 mL aliquots of this suspension were pipetted into sterile test tubes (13 × 100 mm) containing 2 mL of sterile SM buffer and then 5×10^5 bacteriophage particles were added to each test tube. The bacteriophage and *E. coli* cells were then incubated at 37°C for 5 minutes to allow the bacteriophage to adsorb. Following the incubation, each suspension was used to inoculate a 2 L flask containing 500 mL of prewarmed (37°C) 2 × YT broth. The cultures were incubated for approximately 5 hours at 37°C with constant shaking at 300 rpm. During the incubation, the OD_{600} of the cultures was monitored to determine when the bacteriophage had caused complete cell lysis to occur. When lysis occurred, 7 mL of chloroform was added to each flask and the incubation continued for 10 minutes. The lysed cultures were then cooled to room temperature before the addition of 2.5 mL of RNase A (0.2 mg/mL) and 0.3 mL of DNase I (1 mg/mL) to each flask. The flasks were left at room temperature for 30 minutes to allow the digestion of the *E. coli* nucleic acids to occur. Sodium chloride was then added to each flask to a final concentration of 1 M and the flasks were allowed to stand on ice for 1 hour after the sodium chloride had

dissolved. The culture lysates were centrifuged at 11 000 × g for 10 minutes at 4°C and the cell-free supernatants pooled in a clean Erlenmeyer flask. Polyethylene glycol 8000 was added to a final concentration of 10% (w/v) to the supernatant and dissolved. After the polyethylene glycol 8000 had dissolved, the suspension was left at 0°C overnight to allow the bacteriophage to aggregate. The aggregated phages were collected by centrifugation at 11 000 × g at 4°C for 10 minutes and the supernatant discarded. The phage pellet was resuspended in 8 mL of SM buffer and combined with an equal volume of chloroform, then this suspension was mixed by repeated inversion and the phases separated by centrifugation at 1600 × g for 10 minutes. The aqueous phase was recovered and 0.5 g of cesium chloride was added per mL of this bacteriophage suspension. The bacteriophage were further purified by centrifugation on a cesium chloride step gradient in an SW 40 rotor at 22 000 rpm for 2 hours at 4°C. The bluish bacteriophage band was collected from the gradient by puncturing the tube with an 18-gauge needle.

2.3 Plasmid isolation and purification

Small scale plasmid DNA preparations were isolated from *E. coli* cells by alkaline extraction (Birnboim, 1983). A 1.5 mL aliquot of an overnight *E. coli* culture was placed in a micro-centrifuge tube and sedimented for 1 min at 13 000 × g. The supernatant was removed by aspiration and the cell pellet was resuspended in 100 µL of ice-cold glucose buffer (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose). Then 200 µL of lysis solution (0.2 M NaOH, 1% SDS) was added to the micro-centrifuge tube and the cell suspension mixed by inversion. After the cell suspension was lysed 150 µL of ice-cold 5 M potassium acetate solution was added and the cell suspension mixed by inversion. After 5 minutes on ice, the cell suspension was centrifuged at 13 000 × g for 5 minutes at 4°C. Then 400 µL of supernatant was transferred to a clean micro-centrifuge tube and mixed with 500 µL of phenol:chloroform (1:1) on a vortex mixer for 30-45 seconds. The micro-centrifuge tube was centrifuged at 13 000 × g for 5 minutes to separate the aqueous and

organic layers, then the aqueous layer was transferred into another microcentrifuge tube and mixed with 1 mL of 95% ethanol. This tube was then placed at -20°C for at least 15 minutes before the plasmid DNA was pelleted by centrifugation at 13 000 × g. After centrifugation the ethanol was removed by aspiration and the DNA pellet was dried under vacuum. It was redissolved by the addition of 100 µL of 10 mM Tris-HCl pH 8.0, 1 mM EDTA (TE) buffer. The redissolved DNA was then treated with 5 µL of RNase A (10 mg/mL) at 37°C for 30 minutes. Plasmid DNA solutions were routinely quantified using a fluorimeter, and 5 µL samples were run on a 0.75% agarose electrophoresis gel to monitor DNA quality. For large scale plasmid preparations, a neutral SDS lysis was performed with 250 mL of *E. coli* culture and the plasmid DNA obtained further purified by isopycnic centrifugation in cesium chloride gradients containing ethidium bromide (Maniatis *et al.*, 1982).

2.4 Restriction enzyme digests

The restriction endonuclease digests were performed with at least one unit of enzyme per µg of DNA, using commercially prepared buffers, and incubation for 2-3 hours at the temperature specified by the supplier.

2.5 Nucleic acid labeling

DNA fragments were usually labeled by the random primer method as described by Feinberg and Vogelstein (1983, 1984). Oligonucleotide probes were labeled with T4 polynucleotide kinase and [γ -^{32}P]-ATP as described by Maxam and Gilbert (1980). DNA size markers, prepared by digestion of λ DNA with either *Bst*EII, *Cla*I, or *Hin*dIII, were radioactively labeled by filling in recessed ends using Klenow with [α-^{32}P]-dATP and nonradioactive CTP, TTP, and GTP (Maniatis *et al.*, 1982). All labeled DNAs were passed through either a Sephadex G-50 or G-25 column to remove unincorporated radioactive nucleotides.

RNA size markers were prepared with T4 RNA ligase by labeling *E. coli* 5S rRNA and yeast $tRNA^{Phe}$ with cytidine 3', 5'-[5'-^{32}P]-bisphosphate ([5'-^{32}P]-pCp) (England and Uhlenbeck, 1978). The RNA labeling reactions were performed at 37°C for 45 minutes with 2.5 units of T4 RNA ligase and [5'-^{32}P]-pCp, which was synthesized by the transfer of the terminal phosphate group of [γ-^{32}P]-ATP to the 5' hydroxyl group of cytidine 3'-monophosphate by T4 polynucleotide kinase.

2.6 Transformation and Transfection

Transformations were performed using competent cells prepared according to either Morrison (1979) or Chung *et al.* (1989). Competent cells were stored at -80°C and thawed on ice just prior to use. Once thawed, a 200 µL aliquot of competent cells was combined with the DNA in a sterile 13×100 mm test tube and left on ice for a minimum of 40 minutes. The tube was then put in a 42°C heating block to give the cells an 80 second heat shock. The heat shock step was omitted when the competent cells used were prepared according to Chung *et al.* (1989). Immediately following the heat shock, the tube was cooled on ice for 2 minutes, and 1 mL of sterile 2× YT medium was added to the tube. The cells were incubated on a tube roller for 1 hour, then 200 µL aliquots were plated on 2× YT plates containing 100 µg/mL ampicillin (amp). While plating the transformed cells, X-gal and IPTG were added if blue/white screening was possible by α-complementation between the vector and host *E. coli* strain.

The procedure described by Morrison (1979) was also used to transfect *E. coli* LE 392 with purified bacteriophage DNA in order to amplify viable λHtM2, λHtM4, and λHtM6. The only modification was the replacement of 1 mL of sterile 2× YT medium with 3 mL of soft agar overlay, which was mixed with the transfected cells and poured onto a prewarmed agar plate.

2.7 Unidirectional deletions with Exonuclease III

Unidirectional deletions of recombinant plasmids were performed using exonuclease III as described by Henikoff (1987). The resulting plasmids were screened by agarose gel electrophoresis to select deletions based on their sizes. The DNA in these agarose gels was then transferred onto nylon membranes (Rigaud *et al.*, 1987) and probed with M13 universal sequencing primer. Plasmids that varied from one another in size by approximately 300 bp and that had an intact primer binding site were used as sequencing templates.

2.8 DNA sequencing

The sequences of the tDNA clones were determined in both orientations using the dideoxy chain termination method (Sanger et el., 1977). Dideoxy sequencing was performed as a two-step reaction, consisting of an extension/labeling reaction followed by a termination reaction (Tabor and Richardson, 1987), that had been modified for use with *Thermus aquaticus* DNA polymerase (Innis *et al.*, 1988). Sequencing reactions, performed on either single-stranded or double-stranded DNA templates, were separated on 6% denaturing polyacrylamide (38:2, acrylamide:N, N'-methylene bisacrylamide) gels. The separated sequencing reaction products were visualized by 8-24 hours of autoradiography at -20°C. The DNA sequencing results were analyzed using the PCGENE DNA analysis software (Intelligenetics Inc.).

Single-stranded plasmid DNA templates were obtained by growing cells from a single *E. coli* colony, which harboured the recombinant plasmid, with 0.2 mL of concentrated M13KO7 helper phage stock in 10 mL of 2× YT broth containing 150 mg/mL amp at 37°C on a tube roller (Vieira and Messing, 1987). After an hour of incubation, kanamycin was added to a final concentration of 70 μg/mL and the culture returned to the tube roller at 37°C for an overnight incubation (12-16 hours). Single-

stranded DNA was extracted from the overnight culture supernatant as described by Messing (1983).

Double-stranded plasmid DNA templates were obtained as described by Birnboim (1983) and further purified by isopycnic centrifugation in cesium chloride gradients (Maniatis *et al.*,1982). These plasmid DNA templates were alkali-denatured (Chen and Seeburg, 1985) prior to sequencing by the dideoxy chain termination method.

DNA sequencing was also performed using an Applied Biosystems model 373A DNA sequencer, primarily following the manufacturer's suggestions. This technology utilizes either fluorescent dye-terminators or dye-labeled primers to detect terminated products.

2.9 Southern Cross experiment

In order to detect identical regions on the recombinant λ bacteriophages Southern cross experiments were performed (Keen *et al.*, 1988). One recombinant λ phage was digested with restriction endonuclease *Hin*dIII, the products were separated electrophoretically on a 0.75% agarose gel with one well 15 cm across, and transferred onto nitrocellulose. Another recombinant λ bacteriophage was digested with restriction endonuclease *Hin*dIII and the resulting fragments radioactively labeled by filling in the recessed 3' ends with [α-^{32}P]-dATP. The labeled DNA was also fractionated on a 0.75% agarose gel with one well 15 cm across. This fractionated DNA was transferred onto the same nitrocellulose membrane after appropriate blocking, perpendicular to the unlabeled DNA. The second transfer was performed under conditions such that the labeled DNA would only remain bound by annealing to the immobilized fragments of the first bacteriophage. This second transfer used a Southern apparatus pre-equilibrated at 37°C and a transfer solution of 50% formamide, 3 × standard saline phosphate EDTA (SSPE), 2.5 × Denhardt's solution and was conducted overnight (i.e. 12-16 hours). The points at

which the labeled DNA annealed to the immobilized DNA were visualized by autoradiography.

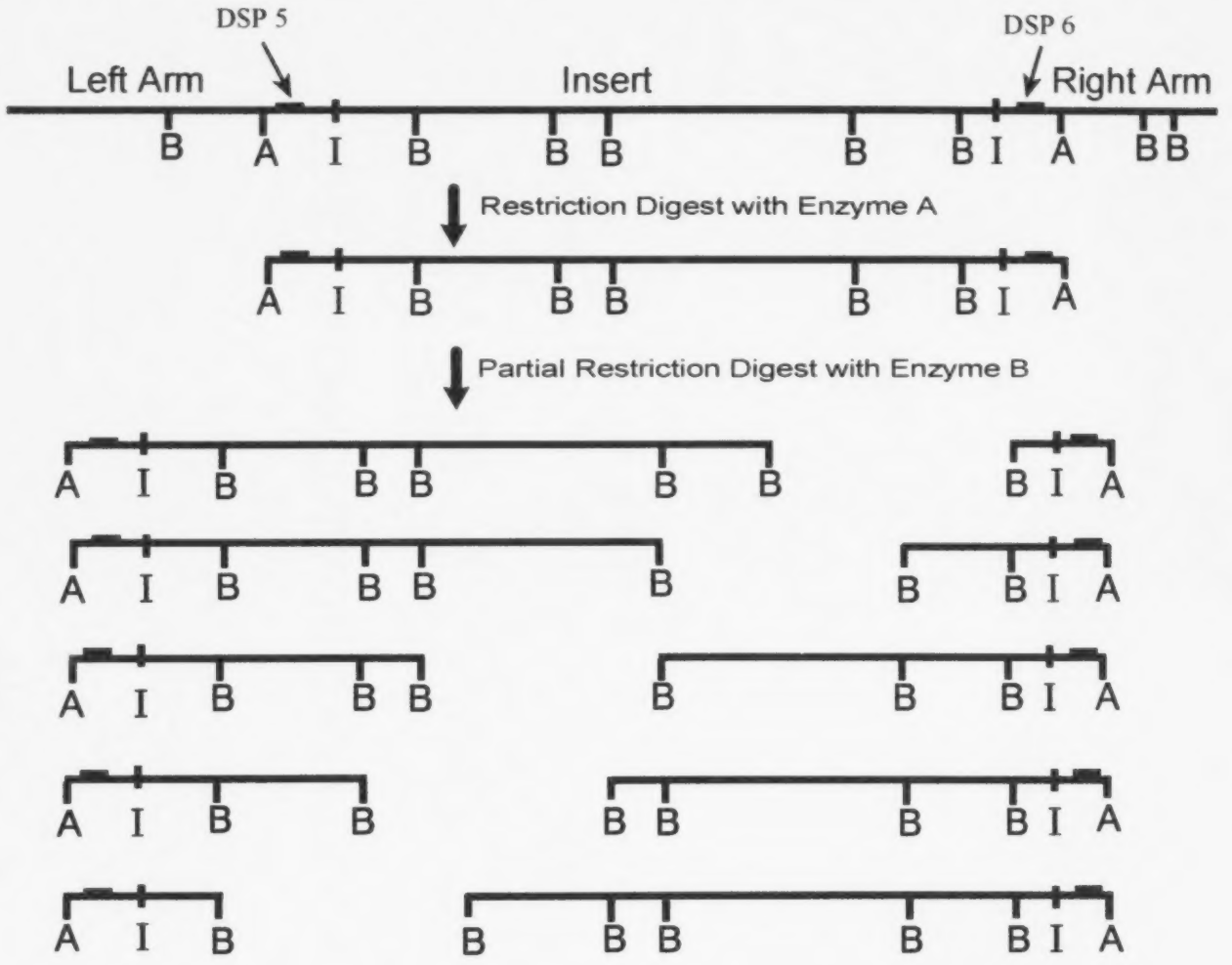
2.10 Restriction endonuclease mapping of recombinant bacteriophages λHtM2, λHtM4, and λHtM6

Restriction endonuclease maps of λHtM2, λHtM4, and λHtM6 were determined using a partial restriction digest mapping technique modified from the strategy first described by Rackwitz *et al.* (1984). This technique is illustrated in Figure 5. The major modification is that the recombinant bacteriophage DNA was first digested with one or more restriction endonucleases that would release the insert DNA with as little vector DNA attached as possible and without also cutting the insert DNA. The digested DNA was precipitated with alcohol, redissolved in TE buffer, and divided into aliquots prior to digestion with a second restriction endonuclease. Each sample of digested DNA was treated with a doubling dilution of restriction enzyme ($1\text{-}^{1}/_{32}$nd unit per tube) in order to achieve the proper partial digest pattern necessary to map the restriction sites. The partially digested DNA samples were electrophoretically separated on a 0.75% agarose gel, in TEA buffer (20 mM Tris-HCl, 50 mM NaOAc, and 2 mM EDTA pH 7.8), which was later stained with ethidium bromide, photographed, and transferred onto a nylon membrane. The Southern transfer was probed twice at 50°C with oligonucleotides specific for lambda sequences, one specific for the right arm and the other specific for the left arm, bordering the insert DNA. The autoradiographs generated from the hybridizations of the Southern transfer allow the restriction sites of the second enzyme to be mapped within the insert DNA by simply determining the sizes of the DNA fragments that hybridize with each oligonucleotide.

These oligonucleotide probes (DSP 5 and DSP 6) were derived from the wild-type lambda sequence (Daniels *et al.*, 1983) and can be used to map recombinant

Figure 5. Schematic diagram of the partial digest restriction mapping technique.

This diagram illustrates how a recombinant λ phage can be mapped with minimal interference from the vector sequences. This recombinant λ phage consists of a DNA fragment cloned into restriction site I of the vector. The first digestion with restriction endonuclease A removes as much of the vector sequences as possible, leaving the insert DNA intact. The second digestion with restriction endonuclease B is performed under conditions favoring partial digestion. The figure only shows the restriction fragments that would be visualized by autoradiography after sequential hybridizations with the left and right oligonucleotide probes. The left arm-specific probe is DSP 5 (TCACCGTGACCGATGACCAT), while the right arm-specific probe is DSP 6 (CCGATAGACCTTACAGTG). The other restriction fragments are present; however, their lack of binding sites for the mapping oligonucleotide prevents them from interfering with the restriction map.


DSP 5
DSP 6
Left Arm
Insert
Right Arm
Restriction Digest with Enzyme A
Partial Restriction Digest with Enzyme B

bacteriophages constructed with either Charon 4A or EMBL 3 lambda vectors. The left arm probe, DSP 5, is a 20-mer (18 569, TCACCGTGACCGATGACCAT, 18 588) and the right arm probe, DSP 6, is an 18-mer (35 184, CCGATAGACCTTACAGTG, 35 201).

2.11 Analysis of the λHtM6 tRNA^{Tyr} gene cluster by PCR

The orientations of the 4 tRNA^{Tyr} genes on λHtM6 were not determined by DNA sequencing alone because the genes are spread over an approximately 10 kb region. Instead of DNA sequencing, PCR amplification (Kleppe *et al.*, 1971; Saiki *et al.*, 1988) of the sequences between the genes was performed to determine both the location and orientation of the 4 tRNA^{Tyr} genes on λHtM6. A total of 12 oligonucleotide primers were used in a series of PCR reactions that generated a collection of PCR products that spanned the entire length of the λHtM6 insert DNA. The 12 primers included 2 gene-specific primers for each tRNA^{Tyr} gene, 2 general tRNA^{Tyr} gene primers, and 2 lambda specific primers:

DSP 1 (CCTTCGATAGCTCAGCTGGTAGAG), tRNA^{Tyr}-R;

DSP 2 (TCCTTCGAGC($^{C}/_{T}$)GGAAT($^{C}/_{T}$)GAACCAG), tRNA^{Tyr}-L;

DSP 5 (TCACCGTGACCGATGACCAT), left λ arm;

DSP 6 (CCGATAGACCTTACAGTG), right λ arm;

DSP 19 (GTCCACAAACGTTTCCGCAGT), 6-2 intron;

DSP 20 (GTCCGCAAATGTCTGTACAAT), 6-1 intron;

DSP 21 (GTCCGCAAATGTCTATACAAT), 6-3 intron;

DSP 22 (GTCCACAAATGTTTCTACAGG), 6-4 intron;

KLR 77 (GCATGCAATGCCACCTGGTGCT), 6-1 3' end;

KLR 78 (ACACGCACGCACCAAAACTACG), 6-4 3' end;

KLR 79 (AGCGCCTGACTCTTTTGCGCAC), 6-2 3' end;

KLR 80 (AAAGCCCTGCAGCTTCCAAGTA), 6-3 3' end.

The PCR reactions were performed in 2 stages. The first stage consisted of 15 cycles involving a denaturation step at 95°C for 45 seconds, an annealing step at 55°C for 45 seconds, and an extension step at 72°C for 4 minutes. The second stage was identical to the first, except for the omission of the annealing step at 55°C for 45 seconds. Each reaction was performed in a 100 µL volume with 70 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 0.1% Triton X-100, 1 unit of Taq DNA polymerase, 0.01 µg of λHtM6, 30 pmoles of each primer, and 0.3 mM deoxyribonucleoside triphosphates. Aliquots from each PCR reaction were fractionated electrophoretically on a 1.0 % agarose gel. Once the gels had been stained with ethidium bromide and photographed, they were transferred onto nylon membrane and hybridized sequentially with different $tRNA^{Tyr}$ gene-specific probes. The autoradiographs of these gels made it possible to locate and orient the $tRNA^{Tyr}$ genes on λHtM6.

2.12 Cloning of PCR amplified $tRNA^{Tyr}$ genes

In order to obtain $tRNA^{Tyr}$ gene-containing plasmid constructs lacking all native flanking sequences, $tRNA^{Tyr}$ gene sequences were amplified by PCR and these PCR products were cloned into pBS. The primers used to amplify the $tRNA^{Tyr}$ genes, DSP 1 and DSP 2, ensured that the principal PCR products generated would not contain any native flanking sequences. The PCR reactions were performed in a 100 µL volume with 70 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 0.1% Triton X-100, 1 unit of Taq DNA polymerase, 0.01 µg of a $tRNA^{Tyr}$ gene-containing plasmid, 50 pmoles of each primer, and 0.3 mM nucleotides. The reactions consisted of 25 cycles involving a denaturation step at 95°C for 45 seconds, an annealing step at 55°C for 45 seconds, and an extension step at 72°C for 1 minute. Aliquots from each PCR reaction were fractionated electrophoretically on a 1.0 % agarose gel, which was later stained with ethidium bromide and photographed. The PCR products, which were chloroform extracted and alcohol precipitated, were cloned into pBS which had been digested either with restriction enzyme

*Hin*dII (or *Hin*cII) or restriction enzyme *Eco*RI followed by treatment with Klenow fragment, dATP and dTTP to generate blunt ends. The ligation reactions were performed in a volume of 10 μL with 50 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 0.5 units of T4 DNA ligase, 0.001 μg of digested pBS, and an aliquot of PCR product for 16 hours at 11°C. Following the 11°C incubation, the ligation reactions were used to transform competent *E. coli* MV 1193 cells. The transformed *E. coli* cells were plated onto 2 × YT plates containing amp, X-gal, and IPTG and the resulting white colonies were further analyzed by colony hybridization with a $tRNA^{Tyr}$ gene-specific probe. Positive clones were sequenced to ensure that the plasmid constructs contained an intact $tRNA^{Tyr}$ gene lacking all native flanking sequences.

2.13 Colony hybridization

Transformants were picked from an antibiotic-containing plate with sterile toothpicks and plated onto two 2 × YT plates containing amp, one of which had a nylon membrane on the agar. When there was sufficient growth on both plates, the colonies that had grown on the nylon membrane were lysed and their denatured DNA baked onto the membrane to allow hybridization with a radiolabeled probe.

The colony lysis was carried out by first placing the nylon membrane, colony side up, on Whatman 3MM paper saturated with 0.5 M NaOH for 3 minutes, or until the colonies became translucent. The nylon membrane was then placed on a second Whatman 3MM paper saturated with 1 M Tris-HCl (pH 8.0) for 4 minutes; this step was repeated once more. The nylon membrane was then placed on a fourth Whatman 3MM paper saturated with 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) for 4 minutes. The nylon membrane was then placed on a fifth Whatman 3MM paper saturated with 2 × SSPE for 4 minutes, and then allowed to dry before being baked at 80°C for 30 minutes. Once the membrane was baked the excess cell debris was washed away using a 0.1% SDS solution, leaving the membrane ready for hybridization with a radiolabeled probe.

2.14 Cell extract preparation for *in vitro* transcription

Cell extracts were made using a procedure adapted from Weil *et al.* (1979), which yields extracts with high levels of RNA polymerase III for *in vitro* transcription experiments. To obtain approximately 2-2.5 mL of cell extract usually 2-3 liters of either HeLa or 293 cell spinner culture, with a density of approximately 5×10^5 cells/mL, were required. The cells were pelleted by centrifugation at 4°C in a Beckman JA 14 rotor at 800 × g for 5 minutes. The cell pellet was resuspended in sterile calcium and magnesium free phosphate-buffered saline and centrifuged at 800 × g to determine the packed cell volume. This step was repeated once more with sterile calcium and magnesium free phosphate-buffered saline and again with 10 volumes of hypotonic buffer (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF), after which the cell pellet was resuspended in 2 packed cell volumes of hypotonic buffer. After leaving the cells in this buffer for 10-20 minutes on ice, the swollen cells were lysed by 13-16 strokes of a Dounce homogenizer. Following cell lysis, a sample of one-ninth the total volume of 0.3 M Hepes (pH 7.9), 30 mM $MgCl_2$, 1.4 M KCl was added to the lysate. The lysate was then centrifuged for 1 hour at 4°C in a Beckman SW 55 rotor at 100 000 × g. The supernatant was collected and a one-fifth volume portion of sterile glycerol was added and the solution was mixed by repeated inversion. Aliquots of cell extract (200 µL) were placed in 0.5 mL centrifuge tubes and quickly frozen in liquid nitrogen and stored at -80°C.

2.15 *In vitro* transcription assays

Transcription assays were performed in 50 µL reaction volumes containing 25 µL of S-100 cell extract. The 50 µL reaction mixtures contained 15 mM Hepes (pH 7.9); 10% (v/v) glycerol; 61 mM KCl; 5 mM $MgCl_2$; 0.6 mM DTT; 0.15 mM PMSF; 1 mM each of ATP, UTP, CTP, and 0.1 mM GTP; 1.0 µCi of [α-^{32}P]-GTP at 3000 Ci/mmol; and 1 pmol of tRNA gene-containing plasmid. Transcription assays were initiated by the

addition of the S-100 cell extract to the other reaction components. Once the reactions were initiated they were incubated at 30°C for 1.5 hours. Following the incubation, the reactions were terminated by the addition of 200 µL of stop mix (6.4 M urea; 0.45 M sodium acetate, pH 5.6; 0.4% SDS; 8 mM EDTA, pH 8.0; and 0.8 µg/mL of yeast RNA) and 200 µL of phenol/chloroform (1:1). Each terminated reaction mixture was vortexed briefly, spun at 13 000 rpm for 1 minute in a MSE Micro Centaur centrifuge, and its aqueous phase collected. The aqueous phase was combined with 1 mL of 95% ethanol and left at -80°C for at least 30 minutes to ensure quantitative precipitation. The extracted nucleic acids were redissolved in 5 µL of formamide dye mix (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, and 2 mM EDTA, pH 8.0) and fractionated electrophoretically on a 10% denaturing polyacrylamide gel with constant power at 32 Watts. The radioactively labeled RNA transcripts were visualized by 8-24 hours of autoradiography at -20°C.

2.16 RNA synthesis *in vitro* with bacteriophage T7 RNA polymerase

RNA was synthesized *in vitro* according to the procedure described by Milligan and Uhlenbeck (1989). The RNA transcripts were synthesized by adding an appropriately linearized DNA template (2-5 µg), containing a T7 promoter sequence, to a reaction mixture with 40 mM Tris-HCl (pH 8.0); 30 mM NaCl; 8 mM $MgCl_2$; 1 mM spermidine; 2.5 mM ATP, UTP and CTP; 0.25 mM GTP; 5.0 µCi of [α-^{32}P]-GTP at 3000 Ci/mmol; and 30 - 60 units of T7 RNA polymerase at 37° - 40°C for 1-2 hours. The synthesized RNA was then precipitated with alcohol, redissolved in formamide dye mix and fractionated electrophoretically on a 15% denaturing polyacrylamide gel to separate the full length transcripts from the prematurely terminated transcripts. The band containing the full length RNA species was excised from the gel and the RNA eluted using an extraction solution (0.5 M NH_4OAc, 0.1 M $Mg(OAc)_2$, 1 mM EDTA (pH 8.0), 0.1%

SDS). The RNA was collected by alcohol precipitation and stored in ethanol until assayed for self-cleavage activity.

2.17 Magnesium ion-promoted RNA self cleavage

The ^{32}P-labeled pre-tRNA transcripts, synthesized either by T7 RNA polymerase or by S-100 cell extracts, were tested for their ability to undergo non-enzymatic intron excision under *in vitro* conditions. The reaction conditions used, which were identical to the conditions described by van Tol *et al.* (1989), involved incubating gel purified tRNA transcripts in 100 mM NH_4OAc (pH 8.0), 10 mM $MgCl_2$, 0.5 mM spermine, and 0.4% Triton X-100 for a minimum of 2 hours at either 37°, 42° or 46°C. These reactions were also conducted including various oligonucleotides (50 pmoles per reaction) that were either identical or complementary to portions of the pre-tRNA transcript. Following the incubation step, the pre-tRNA transcripts were alcohol precipitated and redissolved in formamide dye mix just prior to loading onto a 10% denaturing polyacrylamide gel. After separation by polyacrylamide gel electrophoresis the RNA transcripts were visualized by autoradiography for 3 days at -80°C with a Dupont Lightning Plus intensifying screen.

3. Results

3.1 Restriction endonuclease mapping of λHtM2, λHtM4, and λHtM6

Three recombinant phages were isolated by MacPherson (1988) from a human-λ Charon 4A recombinant bacteriophage library (Lawn *et al.*, 1978), using a DNA fragment containing a $tRNA^{Tyr}$ gene from the 3.18 kb *Xenopus laevis* tRNA gene cluster (Müller and Clarkson, 1980) as a probe. Southern hybridization analysis of these three recombinant bacteriophages, after digestion with several restriction endonucleases, detected a total of six potential human $tRNA^{Tyr}$ genes. Restriction maps, which indicated the $tRNA^{Tyr}$ gene locations within the recombinant bacteriophages, were also generated by MacPherson (1988). Four $tRNA^{Tyr}$ genes were found on λHtM6, named 6-1, 6-2, 6-3 and 6-4, while λHtM2 and λHtM4 each apparently contained one $tRNA^{Tyr}$ gene. These were named M2 and 4-1 respectively.

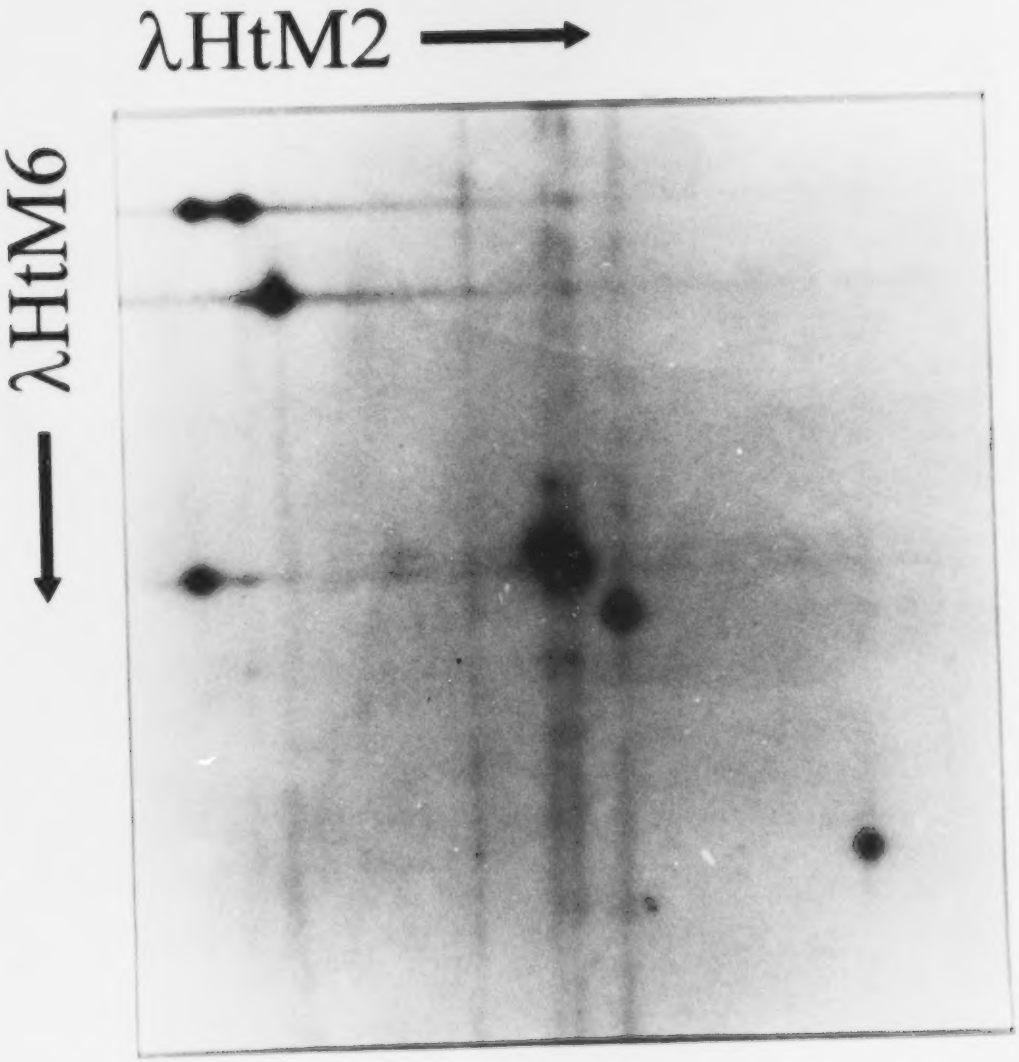
The four $tRNA^{Tyr}$ genes on λHtM6 were isolated on DNA fragments released by the restriction endonuclease *Hin*dIII and cloned into the plasmid vector pAT153 (Twigg and Sherratt, 1980). These recombinant plasmids, which consisted of 1.3, 1.4, 1.5 and 2.0 kb DNA fragments cloned into pAT153, were named pM6IT, pM6, pM6128 and pM612, respectively. The 6-1, 6-2, 6-3, and 6-4 $tRNA^{Tyr}$ genes were carried on plasmids pM6128, pM6, pM612 and pM6IT, respectively. The $tRNA^{Tyr}$ gene on λHtM4, 4-1, was isolated on a 2.4 kb DNA fragment released by a double digest, with restriction endonucleases *Hin*dIII and *Eco*RI, and cloned into pAT153. The resulting recombinant plasmid was named pJM4. Of the six potential $tRNA^{Tyr}$ genes, five were successfully subcloned into plasmid vectors, sequenced, and verified as intron-containing tyrosine tRNA genes (MacPherson and Roy, 1986; MacPherson, 1988). At the commencement of this project the only known $tRNA^{Tyr}$ gene that was not cloned was the one present on λHtM2, tentatively named M2.

To accomplish the main objective of this study, which was the characterization of extragenic sequences that might modulate the transcription of human $tRNA^{Tyr}$ genes, it was necessary to have as many $tRNA^{Tyr}$ genes available as possible for comparative transcription studies. Therefore the cloning and sequencing of the remaining $tRNA^{Tyr}$ gene, M2, from λHtM2 was undertaken. Since the location of the M2 $tRNA^{Tyr}$ gene on λHtM2 had been established by restriction mapping (MacPherson, 1988), a DNA fragment released by double digestion with restriction endonucleases *Hin*dIII and *Bgl*II was chosen for cloning. The resulting recombinant plasmid, named pM2, consisted of a 0.85 kb *Hin*dIII/*Bgl*II DNA fragment cloned into pUC118. The nucleotide sequence of pM2 confirmed the presence of an intron-containing $tRNA^{Tyr}$ gene; however, this sequence was identical to that of pM6128. This unexpected result suggested that λHtM2 and λHtM6 might be overlapping clones, although the restriction maps generated for λHtM2 and λHtM6 (MacPherson, 1988) did not show any clear evidence of an overlapping region.

To determine quickly if λHtM2 and λHtM6 were overlapping clones, Southern cross experiments (Keen *et al.*, 1988) were performed, as described in Materials and Methods section 2.9. The restriction endonuclease *Hin*dIII was used for these experiments because it isolated each of the four $tRNA^{Tyr}$ genes in λHtM6 on a relatively small DNA fragment (1.3 to 2.0 kb in length). The points at which the labeled λHtM2 DNA annealed to the λHtM6 DNA were visualized by autoradiography (Figure 6). These points form a diagonal line across the autoradiograph indicating that certain DNA fragments of λHtM2 and λHtM6 are identical. On the autoradiograph the prominent point that lies above the diagonal is due to a λHtM2 *Hin*dIII fragment annealing to a chimeric λHtM6 *Hin*dIII fragment that contained both λ and human DNA. The prominent point seen below the diagonal is due to the chimeric λHtM2 *Hin*dIII fragment annealing to a λHtM6 *Hin*dIII fragment, which is of entirely human origin.

Figure 6. Southern cross of λHtM2 against λHtM6.

A sample of λHtM6 DNA was digested with the restriction endonuclease *Hin*dIII, fractionated on a 0.75% agarose gel (with one well 15 cm across), and transferred onto nitrocellulose. A sample of λHtM2 DNA was also digested with restriction endonuclease *Hin*dIII, radioactively labeled by filling in the 3' recessed ends with α-^{32}P-dATP, fractionated on a 0.75% agarose gel (with one well 15 cm across) and transferred onto the same nitrocellulose membrane perpendicular to λHtM6. This transfer was performed under hybridization conditions, such that λHtM2 DNA would only bind by annealing to λHtM6 DNA. The direction of electrophoresis of the λHtM2 and λHtM6 DNA fragments is shown by the arrows.


λHtM2
λHtM6

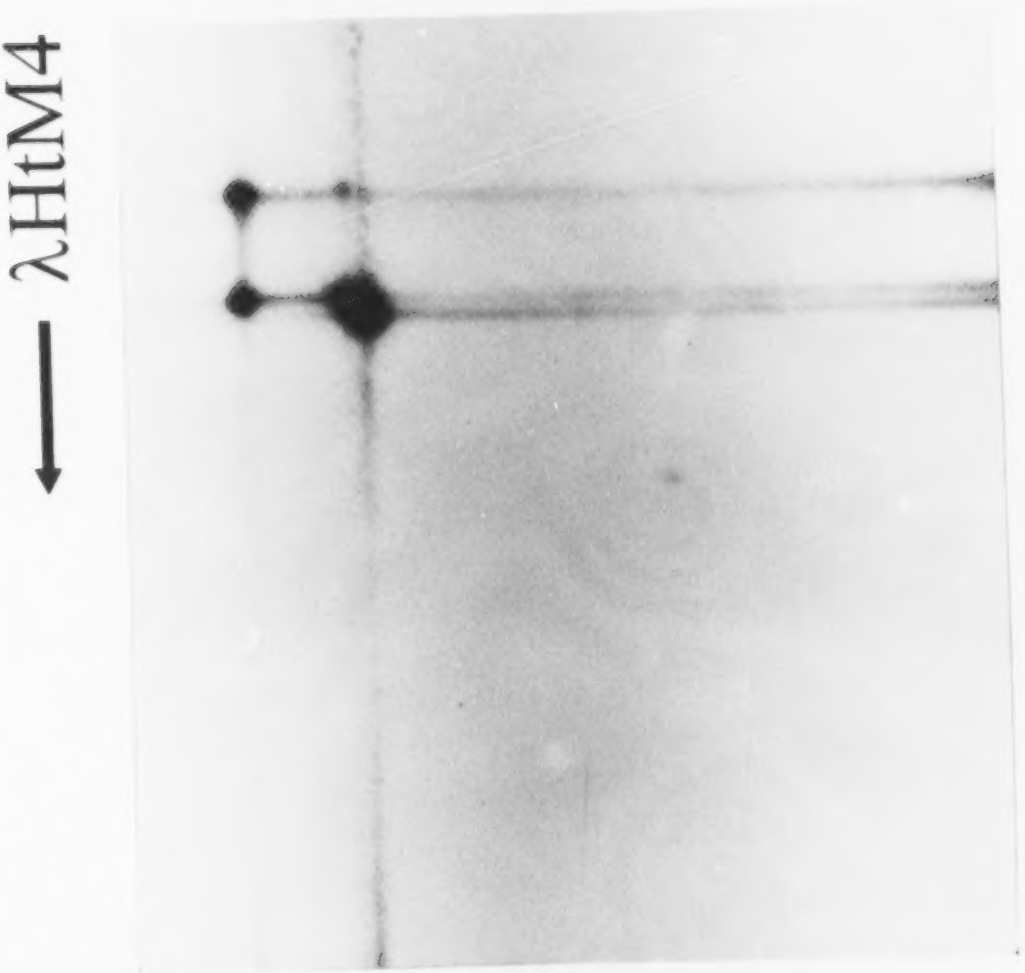
A Southern cross experiment was also performed with λHtM2 and λHtM4 to search for an overlapping region because the restriction maps of these bacteriophage clones appeared similar. However, in this case the only three points seen on the diagonal were the result of λ Charon 4A DNA fragments annealing (Figure 7). The $tRNA^{Tyr}$ gene-containing *Hin*dIII fragment of λHtM2 annealed weakly to the gene-containing *Hin*dIII fragment of λHtM4, however, this point occurred above the diagonal. The prominent point that was below the diagonal was the result of the right λ arm fragment λHtM4 annealing to the left and right λ arm fragments of λHtM2, joined together by the cohesive (cos) ends. Since none of the DNA fragments liberated from the inserts of these bacteriophage clones annealed to give rise to points on the diagonal, it was concluded that the human DNA inserts of λHtM2 and λHtM4 do not overlap.

To identify clearly the overlapping region of λHtM2 and λHtM6, more restriction endonuclease digestions were performed. Digestions of λHtM2, λHtM4 and λHtM6 DNA with restriction endonuclease *Hin*dIII were fractionated electrophoretically on an agarose gel, transferred onto a nylon membrane and hybridized sequentially with λHtM2 DNA and a $tRNA^{Tyr}$ gene-specific oligonucleotide, DSP 1. Hybridization of λHtM2 DNA to the Southern transfer (Figure 8, panel B) identified the 5.7 kb λ DNA fragment common to all three bacteriophages, as well as the larger chimeric human-λ DNA fragments, in lanes 3 and 4. The λHtM2 DNA probe also identified the overlapping fragments between λHtM2 and λHtM6, seen in lanes 3 and 5. These small bands (i.e. less than 2.0 kb) common to λHtM2 and λHtM6 were due to the overlapping region, since similar bands were not detected in λHtM4, seen in lane 4. The smear of low molecular weight species in lane 4 (Panel B and C) is due to RNA contamination of λHtM4 DNA. The $tRNA^{Tyr}$ gene-specific probe identified the 1.5 kb band as the 6-1 $tRNA^{Tyr}$ gene-containing fragment amongst the subset of common DNA fragments (Figure 8, panel C), as well as the other gene-containing fragments. A simultaneous comparison of all the

Figure 7. Southern cross of λHtM2 against λHtM4.

This autoradiograph indicates points where λHtM2 DNA has annealed to the immobilized λHtM4 DNA. A sample of λHtM4 DNA was digested with restriction endonuclease *Hin*dIII and fractionated on a 0.75% agarose gel (with one well 15 cm across) and transferred onto nitrocellulose. An aliquot of λHtM2 DNA was digested with restriction endonuclease *Hin*dIII, radioactively labeled by filling in the 3' recessed ends with α-^{32}P-dATP, fractionated on a 0.75% agarose gel (with one well 15 cm across) and transferred onto the same nitrocellulose membrane perpendicular to λHtM4. The second transfer was performed under conditions such that λHtM2 DNA would bind only by hybridizing to λHtM4. The direction of electrophoresis of the λHtM2 and λHtM4 DNA fragments is shown by the arrows.

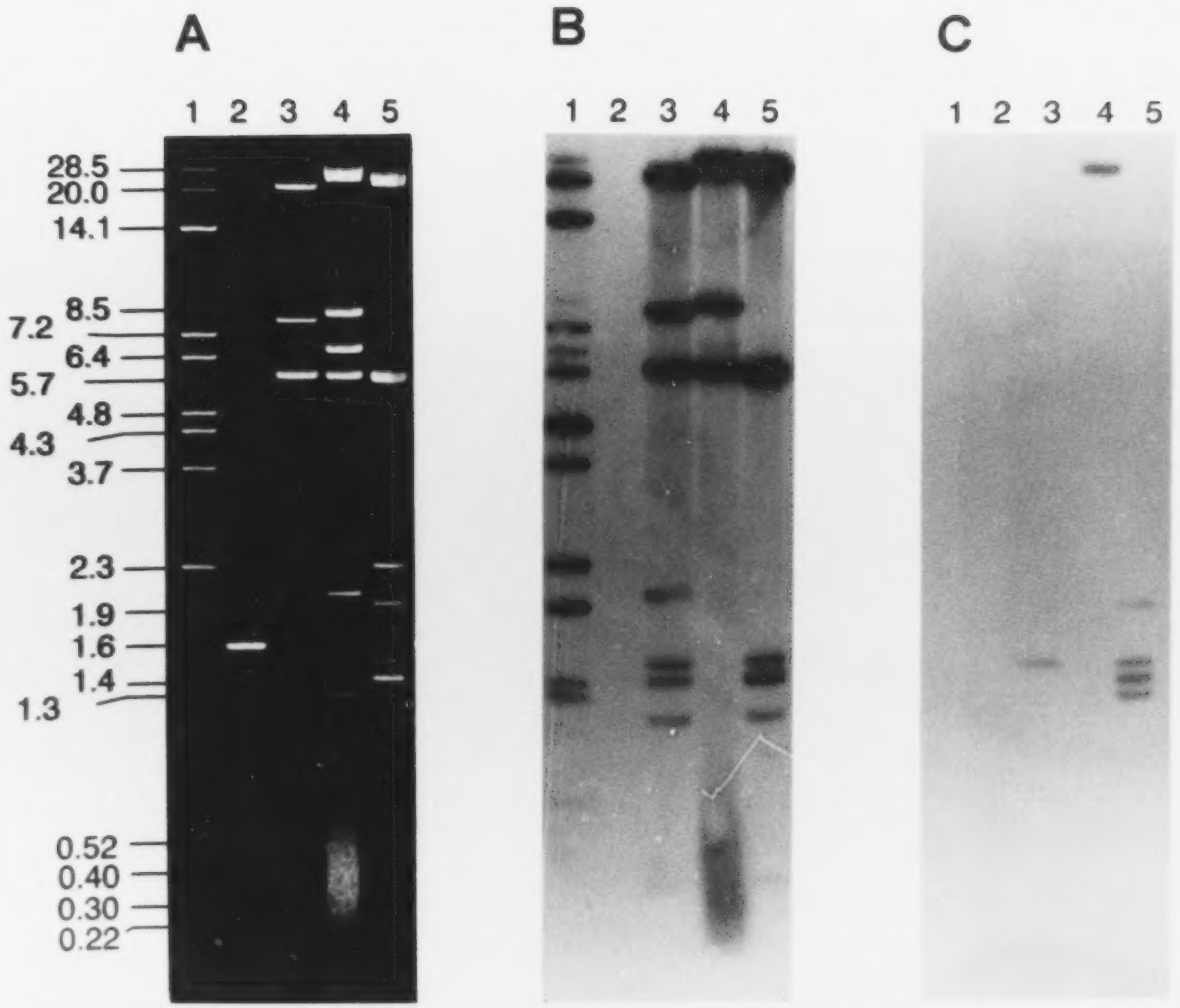
λHtM2 ⟶



⟵ λHtM4

Figure 8. Restriction endonuclease digestion of λHtM2, λHtM4 and λHtM6 DNAs with *Hin*dIII.

Three recombinant lambda DNAs were digested with *Hin*dIII and fractionated on a 1.5% agarose gel. The gel was stained with ethidium bromide and the DNA fragments transferred onto a nylon membrane. Panel A is a photograph of the agarose gel stained with ethidium bromide. Panel B is an autoradiograph of the Southern transfer of the gel probed with radioactively labeled λHtM2 DNA. Panel C is an autoradiograph of the Southern transfer probed with DSP 1, an oligonucleotide specific for $tRNA^{Tyr}$ genes. Lane 1, λ DNA digested with *Cla*I and λ DNA digested with *Nae*I, and lane 2, pAT153 DNA digested with *Hin*fI, are DNA size markers. Lanes 3 - 5 contain *Hin*dIII digested λHtM2, λHtM4, and λHtM6 DNA respectively.


A
B
C
1 2 3 4 5
28.5
20.0
14.1
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.6
1.4
1.3
0.52
0.40
0.30
0.22

panels in Figure 8 revealed that the 1.4 kb λHtM6 DNA fragment in lane 5 is a doublet, consisting of an overlapping fragment and a $tRNA^{Tyr}$ gene-containing fragment.

The previous restriction maps of λHtM2 and λHtM6 (MacPherson, 1988) contained several errors and did not indicate any overlapping region. Therefore corrections to these maps were necessary. To characterize the overlapping region between λHtM2 and λHtM6 further, a comparison of their $tRNA^{Tyr}$ gene-containing DNA fragments was made by digesting them with several restriction endonucleases. The digested λHtM2 and λHtM6 DNAs were fractionated on an agarose gel, transferred to a nylon membrane and hybridized with a $tRNA^{Tyr}$ gene-specific probe (Figure 9, panel B). While all of the $tRNA^{Tyr}$ genes were detected by this probe, comparing the sizes of the 6-1 $tRNA^{Tyr}$ gene-containing fragments, in particular, aided in the assembly of revised restriction maps.

Revisions to the λHtM2 and λHtM6 restriction maps required that more restriction endonuclease digestions be performed. The restriction endonuclease digestion patterns of λHtM2 are shown in Figure 10, which also identifies the 6-1 $tRNA^{Tyr}$ gene-containing fragments. The $tRNA^{Tyr}$ gene-specific oligonucleotide probe DSP 1 also annealed weakly to another site on the Southern transfer of the digested λHtM2 DNA (Figure 10, panel B). However, this secondary binding is not due to another $tRNA^{Tyr}$ gene, since it was not detected when the hybridization temperature was increased from 52° to 56°C or the wash temperature was increased from 47° to 52°C.

To revise the λHtM6 restriction map it was necessary to distinguish the $tRNA^{Tyr}$ genes from one another by sequential hybridizations with probes specific for each individual $tRNA^{Tyr}$ gene. To reduce the chance of one oligonucleotide probe annealing to more than one $tRNA^{Tyr}$ gene, these oligonucleotides were designed to anneal to the 3' flanking sequences immediately downstream of the gene. The specificity of these probes is demonstrated in Figure 11, which shows a Southern transfer of doubly digested λHtM6 DNA hybridized sequentially with each of these specific $tRNA^{Tyr}$ gene probes. Panels B,

Figure 9. Comparison of λHtM2 and λHtM6 restriction fragments carrying $tRNA^{Tyr}$ genes.

Samples of λHtM2 and λHtM6 DNA (2-3 μg) were digested with restriction enzymes and the products separated on a 1.0% agarose gel. The gel was stained with ethidium bromide (A) and the products transferred onto a nylon membrane. The restriction enzymes used to digest the λHtM2 DNA (lanes 2, 4, 6, 8, and 10) and the λHtM6 DNA (lanes 3, 5, 7, 9, and 11) were *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII and *Sty*I, respectively. Lane 1, λ DNA digested with *Bst*EII, serves as the DNA size markers. Panel B is an autoradiograph of the Southern transfer probed with DSP 1, an oligonucleotide specific for $tRNA^{Tyr}$ genes.

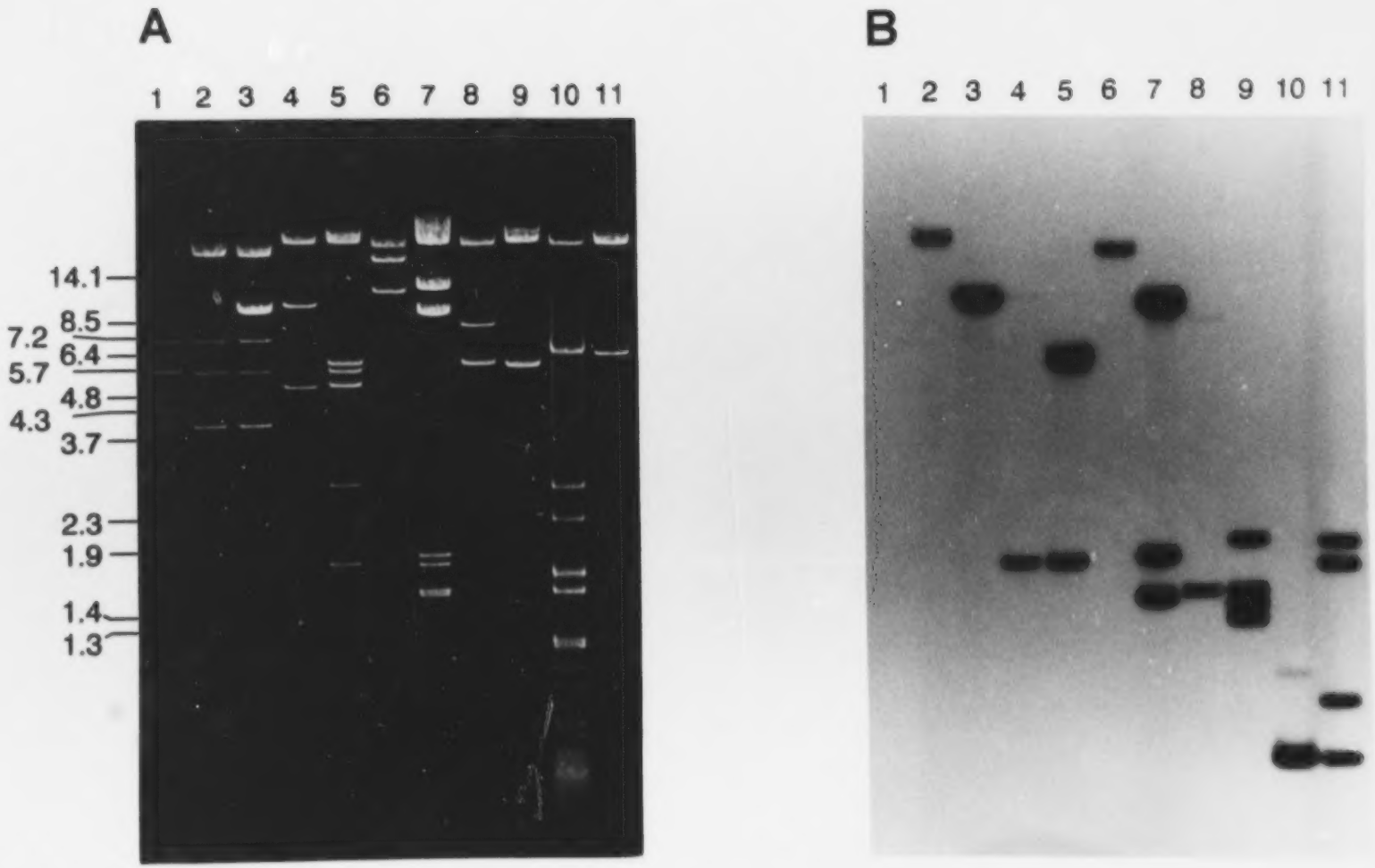

A
1 2 3 4 5 6 7 8 9 10 11
14.1
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.4
1.3
B
1 2 3 4 5 6 7 8 9 10 11

Figure 10. Identification of λHtM2 restriction fragments carrying a $tRNA^{Tyr}$ gene.

Samples of λHtM2 DNA (2 - 3 μg) were digested with restriction enzymes, then separated on a 0.75% agarose gel. The gel was stained with ethidium bromide (panel A) and the DNA was transferred onto a nylon membrane. The restriction enzymes used to digest the λHtM2 DNA in lanes 2 - 5 were *Bam*HI, *Bgl*II, *Eco*RI, and *Hin*dIII, respectively. The λHtM2 DNA in lanes 6 - 9 was doubly digested with *Bam*HI/*Hin*dIII, *Bgl*II/*Hin*dIII, *Bam*HI/ *Eco*RI, and *Eco*RI/*Hin*dIII respectively. Lane 1, λ DNA digested with *Bst*EII, and lane 10, λ DNA digested with *Hin*dIII, are DNA size markers. Panel B is an autoradiograph of the Southern transfer probed with DSP 1, an oligonucleotide specific for $tRNA^{Tyr}$ genes.

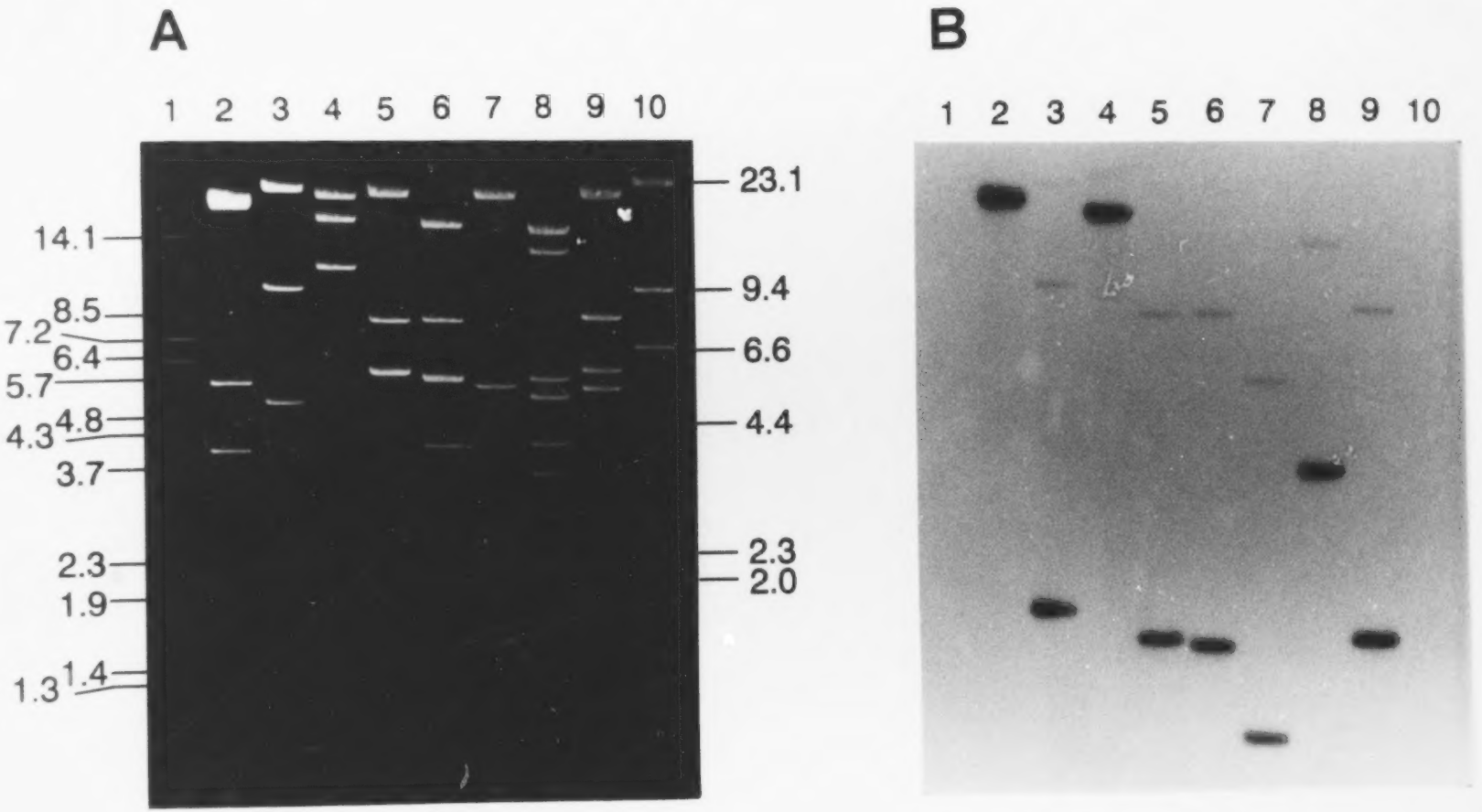
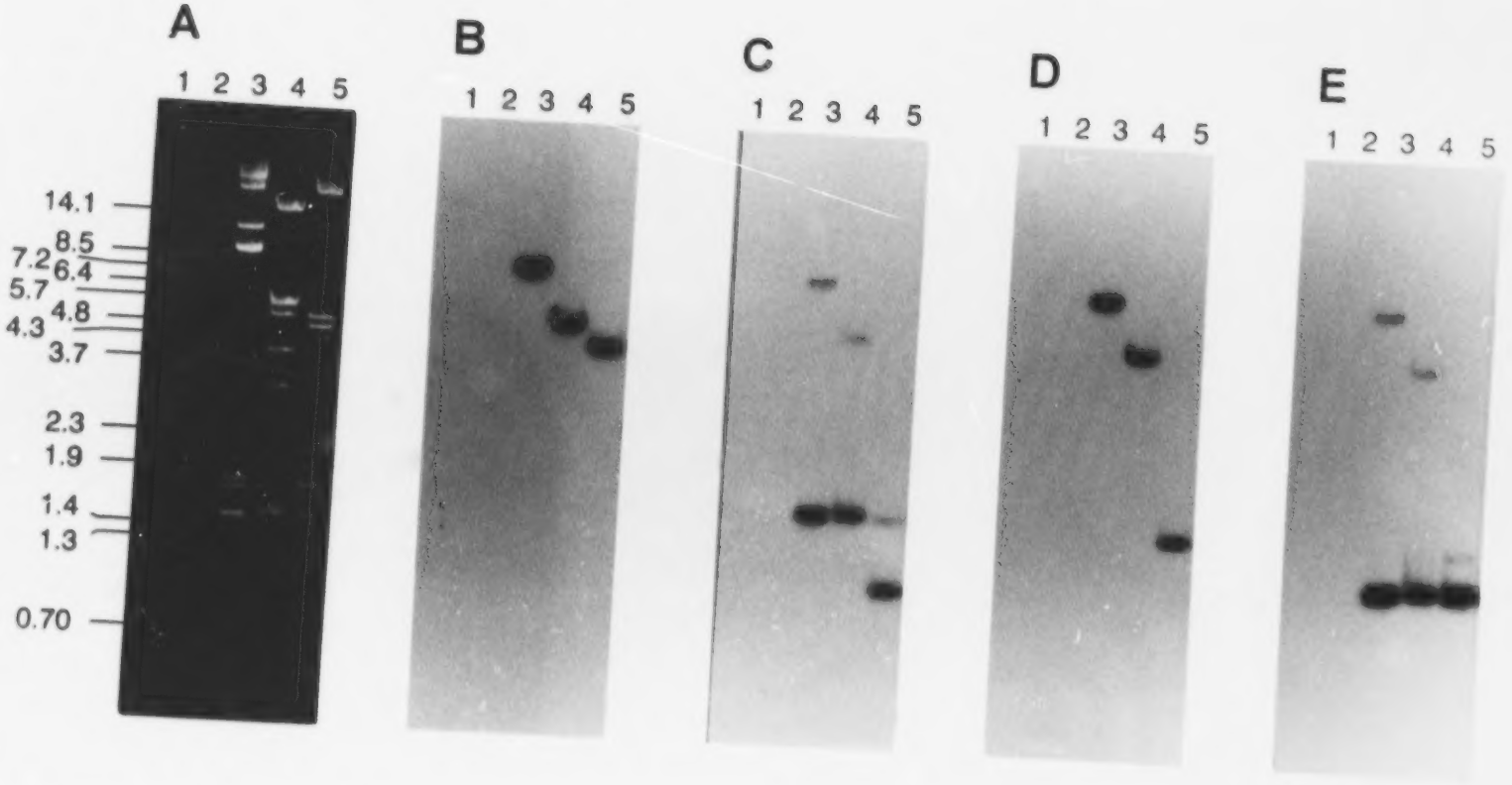

A
1 2 3 4 5 6 7 8 9 10
14.1
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.4
1.3
23.1
9.4
6.6
4.4
2.3
2.0
B
1 2 3 4 5 6 7 8 9 10

Figure 11. Identification of λHtM6 restriction fragments carrying specific $tRNA^{Tyr}$ genes.

Samples of λHtM6 DNA (2 - 3 μg) were digested with restriction enzymes and separated on a 0.75% agarose gel. The gel was stained with ethidium bromide (Panel A) and the DNA transferred onto a nylon membrane. Lane 1, λ DNA digested with *Bst*EII, and lane 2, λ DNA digested with *Cla*I, serve as DNA size markers, although lane 2 is of little use because insufficient DNA was loaded. The restriction enzyme used to digest the λHtM6 DNA in lane 3 is *Eco*RI. The remaining lanes (lanes 4 and 5) contain doubly digested λHtM6 DNA. The DNA was first digested with *Eco*RI and aliquots were then digested with *Bam*HI and *Bgl*II, corresponding to lanes 4 and 5, respectively. Panel B shows the restriction fragments that carry the 6-2 gene, visualized by probing with oligonucleotide KLR 79. Panel C shows the restriction fragments that carry the 6-3 gene, visualized by probing with oligonucleotide KLR 80. Panel D shows the restriction fragments that carry the 6-1 gene, visualized by probing with oligonucleotide KLR 77. Panel E shows the restriction fragments that carry the 6-4 gene, visualized by probing with oligonucleotide KLR 78.


A
1 2 3 4 5
14.1
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.4
1.3
0.70
B
1 2 3 4 5
C
1 2 3 4 5
D
1 2 3 4 5
E
1 2 3 4 5

C, D and E of Figure 11 show the 6-2, 6-3, 6-1 and 6-4 $tRNA^{Tyr}$ gene-containing fragments, respectively. The faint bands seen in lanes 3, 4 and 5 were due to traces of probe from the previous hybridization that were not removed by stripping the nylon membrane. Other Southern transfers of restriction endonuclease digested λHtM6 DNA, previously hybridized with DSP 1, were also hybridized sequentially with each of these four probes to identify specifically each of the four $tRNA^{Tyr}$ genes (data not shown).

Due to the complexity of the λHtM2 and λHtM6 digest patterns, these complete digests alone were insufficient for generating unambiguous restriction maps. For example, the DNA fragments generated by *Hin*dIII digestion of λHtM6 vary in size from 2.4 to 0.15 kb, except for the left and right arm fragments of λ Charon 4A. The data obtained from hybridizing Southern transfers of these digested bacteriophage DNAs with several oligonucleotide probes is summarized in Tables 3 and 4 for λHtM2 and λHtM6, respectively. These Tables list by size the DNA fragments generated by digestion with selected restriction endonucleases. In these Tables the gene-containing fragments are identified by the particular $tRNA^{Tyr}$ gene(s) they carry. When these Tables were combined with data obtained from partial digests of λHtM2 and λHtM6 DNAs the ambiguities in their restriction map were resolved.

Partial digestion was undertaken to aid in the assembly of. Partial digestions of λHtM4 were conducted to serve as controls for mapping since no errors were suspected in its restriction map. A partial mapping strategy was devised for increased resolution by designing oligonucleotide probes closer to the insert DNA. The new probes, DSP 5 and DSP 6, made it possible to remove much of the vector DNA with complete restriction endonuclease digests before continuing with the partial digests. This modification reduces the length of DNA that has to be mapped by partial digestion from 45 kb to approximately 20 kb, which improves the separation of the resulting partially digested DNA fragments by ordinary agarose gel electrophoresis with 0.75% gels. An example of this partial digestion restriction mapping technique is shown in Figure 12, which illustrates the mapping of

Table 3. DNA fragments generated by restriction endonuclease digestion of λHtM2[a].

*Bam*HI	*Bgl*II	*Eco*RI	*Hin*dIII
2 × 17.1♣[b]	21.8	20.0	20.0
5.6	9.65	14.0♣	7.8
3.9	4.8	11.0	2 × 5.7
1.3	2.3		2.0
	2.0		1.5♣
	1.8♣		1.4
	1.3		1.1
	1.0		0.35

[a] The sizes of the restriction fragments are given in kb.

[b] DNA fragments that carry the 6-1 $tRNA^{Tyr}$ gene are indicated with a symbol (♣).

Table 4. DNA fragments generated by restriction endonuclease digestion of λHtM6[a].

*Bam*HI	*Bgl*II	*Eco*RI	*Hin*dIII
18.2	21.2	20.0	21.5
2x 9.2♣♥♠♦	6.25♥♠	11.0	2x 5.7
5.6	5.8♦	9.5♣♥	2.4
3.9	4.8	1.85♠	2.0♠[d]
1.5	2.8	1.75	1.5♣[b]
	2.0	2x 1.5♦	2x 1.4♥[c]
	2x 1.8♣	0.75	1.3♦[e]
	1.3	0.60	1.1
			0.90
			0.85
			0.70
			0.35
			0.15

[a] The sizes of the restriction fragments are given in kb.

[b] The 6-1 $tRNA^{Tyr}$ gene-containing DNA fragments are indicated with a symbol (♣).

[c] The 6-2 $tRNA^{Tyr}$ gene-containing DNA fragments are indicated with a symbol (♥).

[d] The 6-3 $tRNA^{Tyr}$ gene-containing DNA fragments are indicated with a symbol (♠).

[e] The 6-4 $tRNA^{Tyr}$ gene-containing DNA fragments are indicated with a symbol (♦).

*Eco*RI restriction sites on λHtM4. The λHtM4 DNA was first digested with restriction endonucleases *Bam*HI and *Kpn*I to remove the λ Charon 4A vector sequences. This digested λHtM4 DNA was then partially digested with restriction endonuclease *Eco*RI and the cleavage sites mapped by hybridizing the Southern transfer sequentially with DSP 5 and DSP 6.

The three bacteriophage clones were digested with several restriction endonucleases in order to find those that cleave the vector DNA and leave the insert DNA intact. The screening procedure involved digesting λHtM2, λHtM4 and λHtM6 DNAs, electrophoretically fractionating the digestion products on agarose gels, transferring these products to nylon membranes, and hybridizing sequentially with the mapping oligonucleotides (DSP 5 and DSP 6) and with a $tRNA^{Tyr}$ gene-specific oligonucleotide, DSP 1 (data not shown). The hybridization with DSP 1 was performed to determine if the majority of the insert DNA was intact after the first digestion. Some of the restriction endonucleases that were useful for removing the λ Charon 4A vector sequences were *Bgl*I, *Mlu*I and *Sst*II (data not shown).

While searching for restriction endonucleases that would cleave the λ sequences and leave the insert DNA intact, a Southern transfer of digested λHtM4 DNA was hybridized with a $tRNA^{Tyr}$ gene-specific oligonucleotide (DSP 1) and two bands were detected in the lane containing *Apa*I-digested DNA. This observation indicated a potential new $tRNA^{Tyr}$ gene within λHtM4, which was previously thought to contain only an isolated $tRNA^{Tyr}$ gene. The discovery of an uncharacterized $tRNA^{Tyr}$ gene prompted a reexamination of the λHtM4 restriction map. To improve the λHtM4 restriction map, additional restriction endonuclease digestions of λHtM4 DNA were performed, the digestion products fractionated electrophoretically on an agarose gel, and transferred to a nylon membrane. These restriction digests are shown in Figure 13, along with the autoradiograph of the Southern transfer that had been probed with DSP 1. Figure 13 shows that apart from *Apa*I, seen in lane 2, no other restriction endonuclease liberated two

Figure 12. Restriction endonuclease mapping of λHtM4 by partial digestion.

Approximately 2 μg of λHtM4 DNA were digested to completion with restriction endonucleases *Bam*HI and *Kpn*I (neither of which cuts in the human sequence of this recombinant). The digested DNA was collected by ethanol precipitation, redissolved in TE buffer and divided equally amongst five microfuge tubes for partial digestion. These samples were partially digested for 5 minutes at room temperature with 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.0 units of restriction endonuclease *Eco*RI and fractionated electrophoretically on a 0.75% agarose gel in lanes 2 - 7, respectively. Lane 1, which contains both *Bst*EII and *Nae*I digested λ DNAs, is a DNA size marker. Panel A is a photograph of the ethidium bromide stained agarose gel and panels B and C are autoradiographs of the Southern transfer of the digested λHtM4 DNA probed sequentially with DSP 5 (left arm probe) and DSP 6 (right arm probe), respectively.

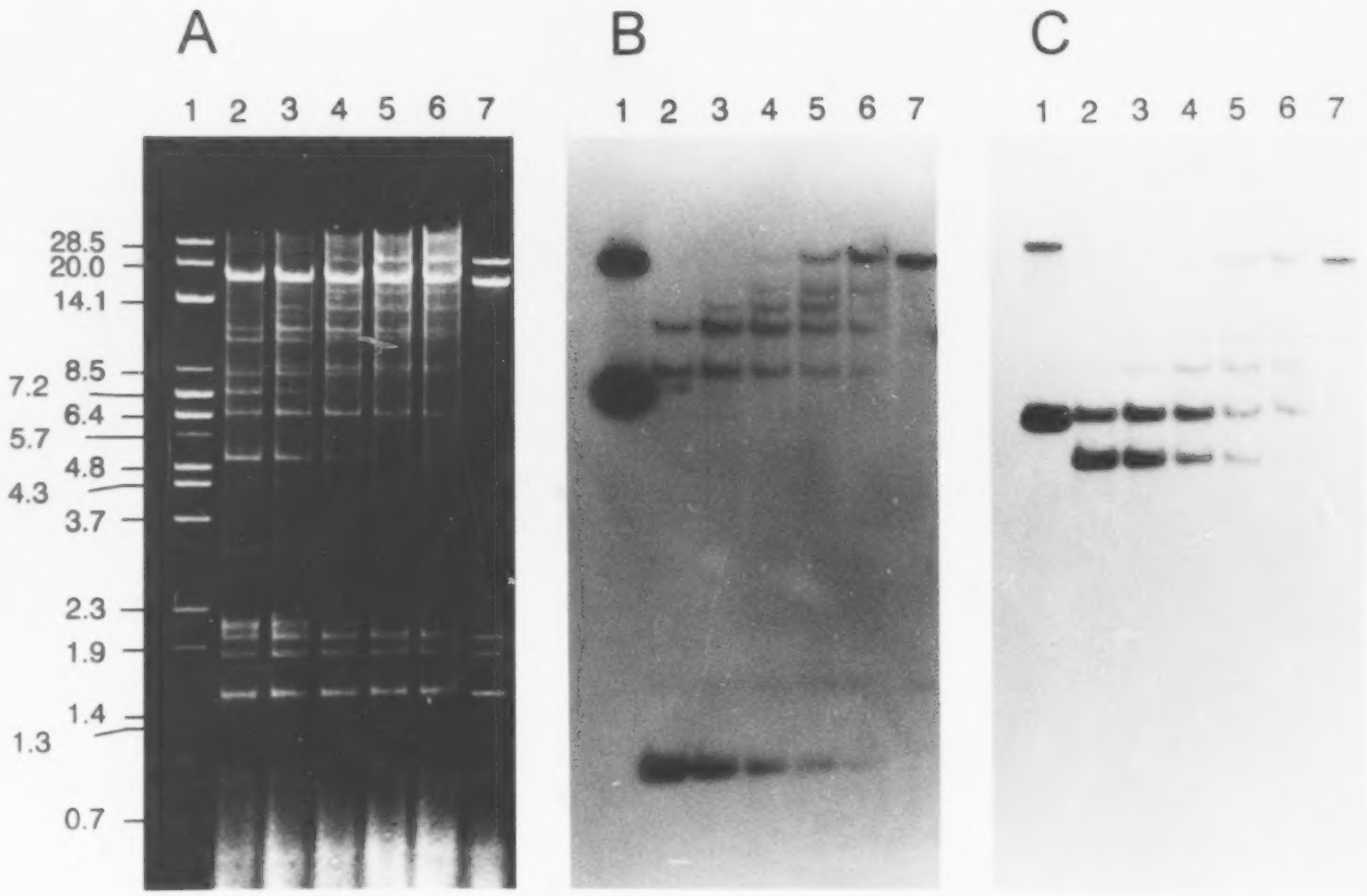
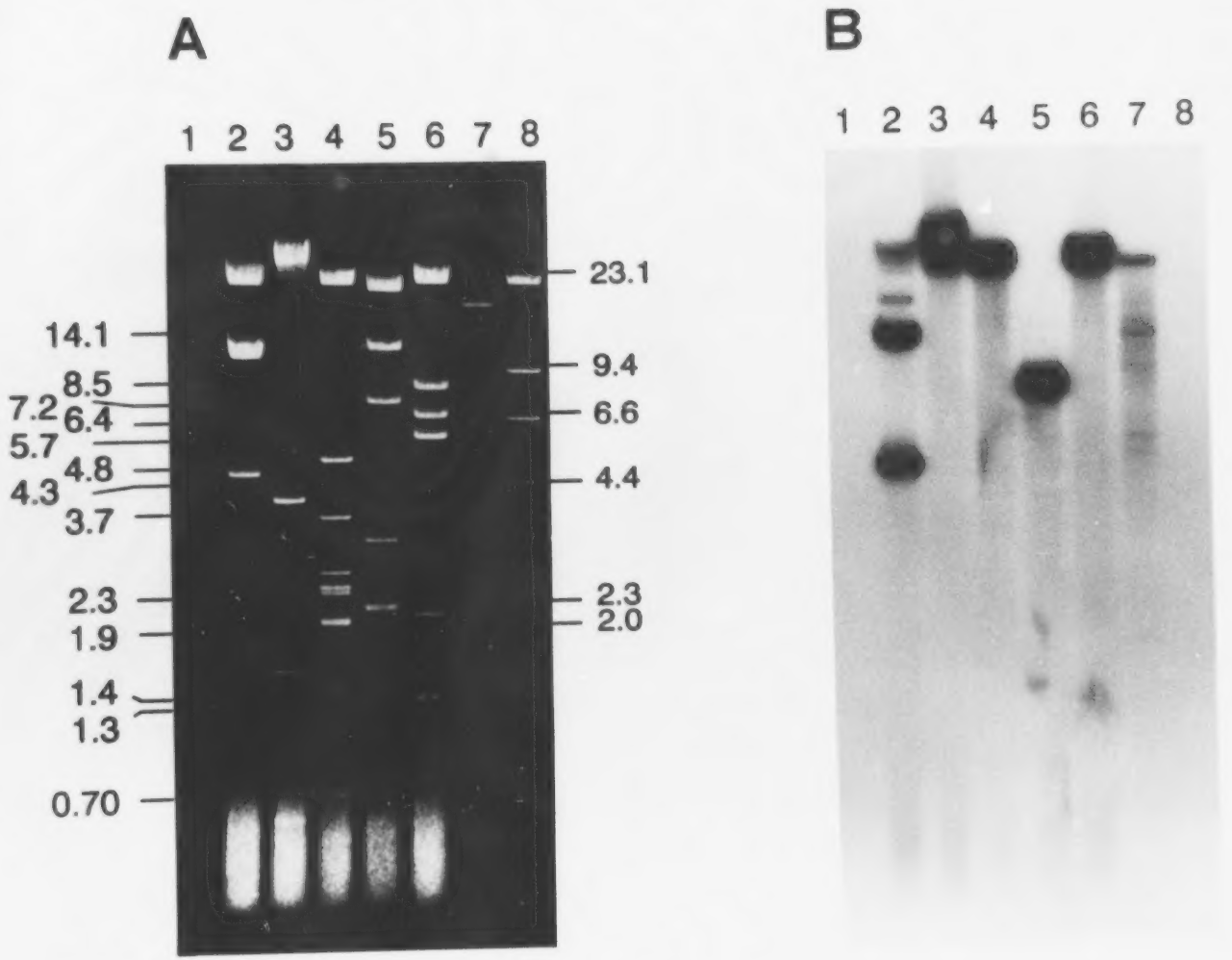

A
B
C
1 2 3 4 5 6 7
28.5
20.0
14.1
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.4
1.3
0.7

Figure 13. Identification of λHtM4 restriction fragments carrying $tRNA^{Tyr}$ genes.

Samples of λHtM4 DNA (2-3 μg) were digested with restriction enzymes and separated on a 0.75% agarose gel. The gel was stained with ethidium bromide (A) and transferred onto a nylon membrane. The restriction enzymes used to digest the λHtM4 DNA in lanes 2 - 7 were *Apa*I, *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, and *Kpn*I respectively. Lane 1, λ DNA digested with *Bst*EII, and lane 8, λ DNA digested with *Hind*III, are DNA size markers. The radioautograph of the Southern transfer (B) shows the restriction fragments that carry a $tRNA^{Tyr}$ gene(s), detected by hybridization with a a $tRNA^{Tyr}$ gene specific oligonucleotide probe (DSP 1).

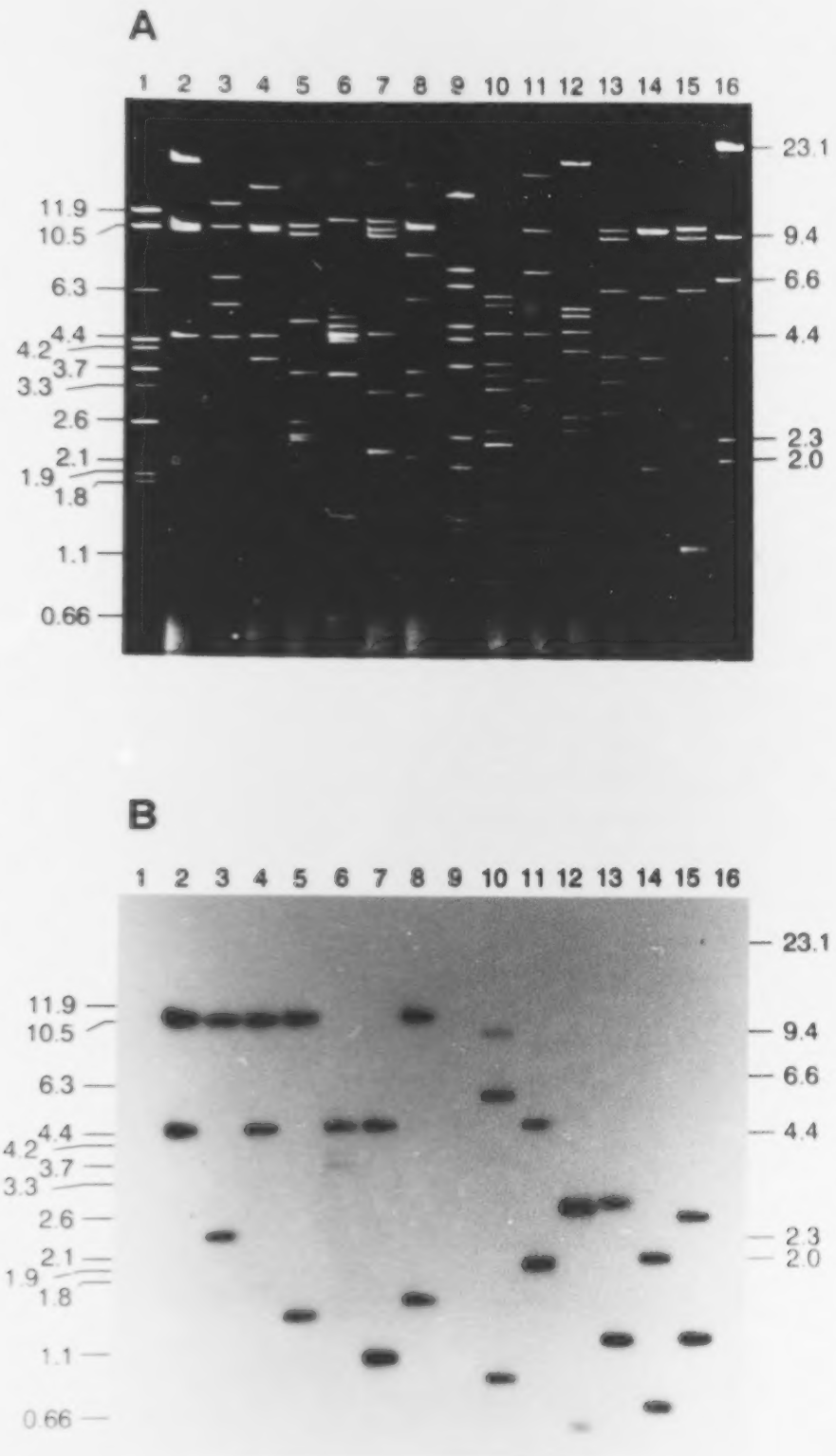

A
1 2 3 4 5 6 7 8
14.1
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.4
1.3
0.70
23.1
9.4
6.6
4.4
2.3
2.0
B
1 2 3 4 5 6 7 8

λHtM4 DNA fragments that annealed with DSP 1. For purposes of gene identification the $tRNA^{Tyr}$ genes found on λHtM4 are referred to as 4-1 and 4-2, which are found on the 4.55 kb and 10.8 kb *Apa*I restriction fragments, respectively. The faint bands seen in lane 2 were probably due to incomplete digestion of λHtM4 DNA by *Apa*I. Unlike the case in lane 2, the faint bands seen in lane 7, were most likely due to overdigestion of λHtM4 DNA by *Kpn*I, with possible star activity. Overdigestion was suspected because all the restriction endonuclease digestions had been performed with equal amounts of λHtM4 DNA, however, significantly less DNA was present in lane 7 (Figure 13, panel A).

In order to obtain DNA fragments suitable for cloning the new $tRNA^{Tyr}$ gene, double restriction endonuclease digestions of λHtM4 and pJM4 (a pAT153 recombinant which contains a 2.4 kb DNA fragment released from λHtM4 by restriction endonucleases *Eco*RI and *Hin*dIII) were performed with *Apa*I in combination with several other restriction endonucleases. The double restriction endonuclease digestion products of λHtM4 and pJM4 DNA were fractionated electrophoretically on agarose gels and transferred to nylon membranes. The results of the λHtM4 DNA double digests are shown in Figure 14, which compares a photograph of the agarose gel containing the electrophoretically fractionated digestion products to an autoradiograph of its corresponding Southern transfer that had been hybridized with DSP 1. On the autoradiograph the faint band seen in lane 6 is probably due to overdigestion by restriction endonuclease *Bss*HII, unlike the faint band seen in lane 10 which seems to be due to incomplete digestion by the restriction endonuclease *Hpa*I. The results of the pJM4 DNA double digests are shown in Figure 15, which compares a photograph of the agarose gel containing the electrophoretically fractionated digestion products to an autoradiograph of its corresponding Southern transfer that had been hybridized with DSP 1. Since both $tRNA^{Tyr}$ genes were contained on pJM4, it was necessary to sequence the entire 2.4 kb insert of this clone to characterize the new $tRNA^{Tyr}$ gene and its surrounding flanking sequences. DNA fragments were cloned, sequenced and a new intron-containing $tRNA^{Tyr}$

Figure 14. Selection of λHtM4 restriction fragments carrying $tRNA^{Tyr}$ genes for subcloning.

Samples of λHtM4 DNA (2 - 3 μg) were digested with restriction enzymes and separated on a 0.75% agarose gel. The gel was stained with ethidium bromide (A) and the products were transferred onto a nylon membrane. Lane 1, λ DNA digested with *Cla*I, lane 9, λ DNA digested with *Bst*EII, and lane 16, λ DNA digested with *Hind*III, are DNA size markers. The restriction enzyme used to digest the λHtM4 DNA in lane 2 is *Apa*I. The other lanes (lanes 3 - 8 and 10 - 15) contain doubly digested λHtM4 DNA. The DNA was first digested with *Apa*I and aliquots were then digested with *Apa*LI, *Bam*HI, *Bgl*II, *Bss*HII, *Eco*RI, *Hind*III, *Hpa*I, *Kpn*I, *Mlu*I, *Nco*I, *Sac*I, and *Sty*I. These double digests correspond to lanes 3 - 8 and 10 - 15, respectively. The radioautograph of the Southern transfer (B) shows the restriction fragments that carry a $tRNA^{Tyr}$ gene(s), detected by hybridization with a $tRNA^{Tyr}$ gene specific oligonucleotide probe (DSP 1).


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
11.9
10.5
6.3
4.4
4.2
3.7
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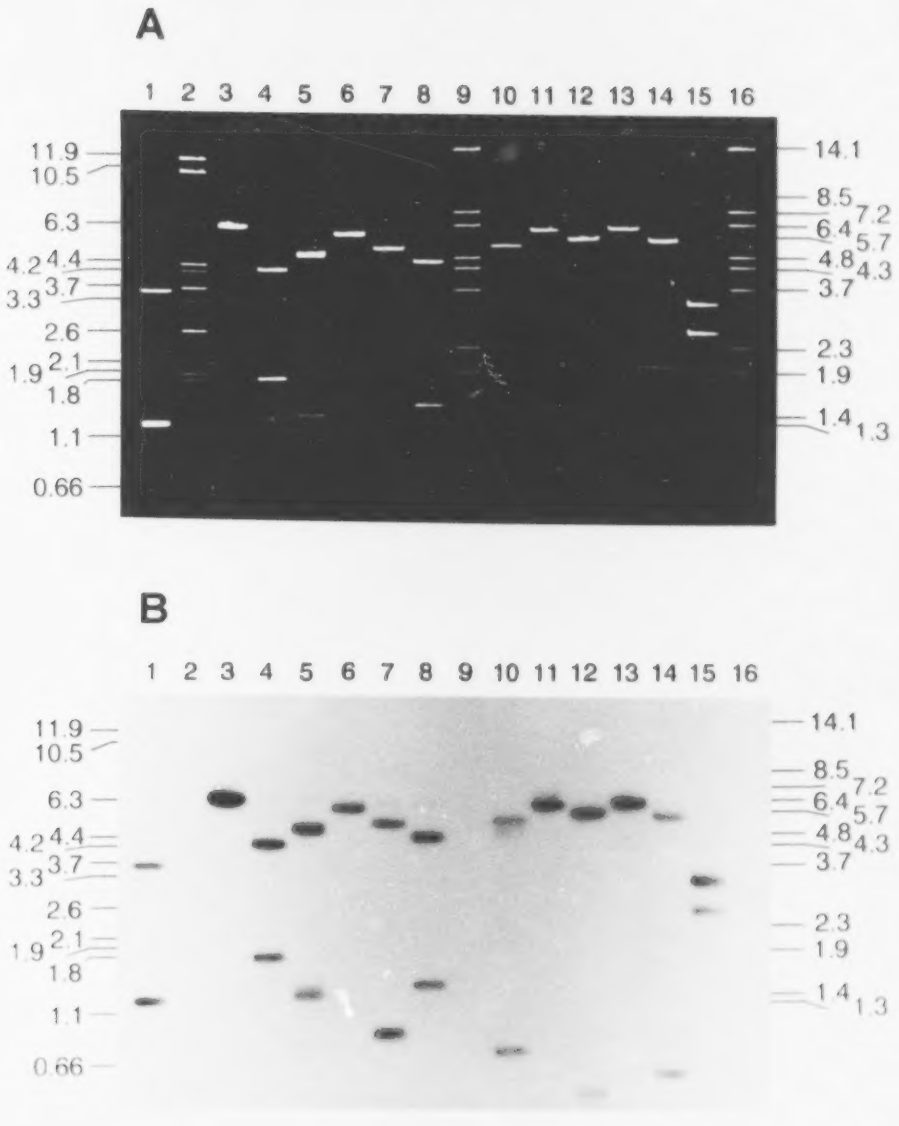
2.6
2.1
1.9
1.8
1.1
0.66
23.1
9.4
6.6
4.4
2.3
2.0
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
11.9
10.5
6.3
4.4
4.2
3.7
3.3
2.6
2.1
1.9
1.8
1.1
0.66
23.1
9.4
6.6
4.4
2.3
2.0

Figure 15. Identification of pJM4 restriction fragments carrying $tRNA^{Tyr}$ genes.

Samples of pJM4 DNA (0.1 - 0.3 μg) were digested with restriction enzymes and separated on a 0.75% agarose gel. The gel was stained with ethidium bromide (A) and the DNA fragments were transferred onto a nylon membrane. Lane 2, λ DNA digested with *Cla*I, and lanes 9, and 16, λ DNA digested with *Bst*EII, are DNA size markers. The restriction enzyme used to digest the pJM4 DNA in lane 3 is *Apa*I. The other lanes (lanes 1, 4 - 8, and 10 - 15) contain doubly digested pJM4 DNA. The DNA was first digested with *Apa*I and aliquots were then digested with *Apa*LI, *Bam*HI, *Bgl*II, *Bss*HII, *Eco*RI, *Hin*dIII, *Hpa*I, *Kpn*I, *Mlu*I, *Nco*I, *Sac*I, and *Sty*I. These double digests correspond to lanes 1, 4 - 8, and 10 - 15 respectively. The radioautograph of the Southern transfer (B) shows the restriction fragments that carry $tRNA^{Tyr}$ genes, detected by hybridization with a a $tRNA^{Tyr}$ gene specific oligonucleotide probe (DSP 1).


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
11.9
10.5
6.3
4.2
4.4
3.3
3.7
2.6
1.9
2.1
1.8
1.1
0.66
14.1
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.4
1.3
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
11.9
10.5
6.3
4.2
4.4
3.3
3.7
2.6
1.9
2.1
1.8
1.1
0.66
14.1
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.4
1.3

gene, 4-2, was characterized, as well as a $tRNA^{Ala}$ gene, 4-3. The nucleotide sequence of this 2.4 kb DNA fragment is provided in section 3.3 of the Results.

The Southern transfers that had been used to detect $tRNA^{Tyr}$ genes on three recombinant bacteriophages were hybridized with $tRNA^{Ala}$ gene-specific probes, but no additional genes were detected (not shown). The data obtained from hybridizing Southern transfers of digested λHtM4 DNA with several oligonucleotide probes is summarized in Table 5.

The restriction endonuclease digestions of λHtM2, λHtM4 and λHtM6, including both complete and partial digests, were used to generate new restriction maps (Figure 16) that indicate the tRNA gene-containing fragments. The specific locations and orientations of the genes on λHtM2 and λHtM4 were determined by sequencing gene-containing DNA fragments. However, the specific locations and orientations of the genes on λHtM6 were determined by DNA sequencing in combination with PCR amplification of sequences between the $tRNA^{Tyr}$ genes. These PCR amplifications are described in the next section.

3.2 Determination of the λHtM6 $tRNA^{Tyr}$ gene orientations by PCR

The restriction map, even combined with the nucleotide sequence of the tRNA gene-containing subclones, was not enough to establish precisely the locations and orientations of the $tRNA^{Tyr}$ genes on λHtM6. The $tRNA^{Tyr}$ gene locations and orientations on λHtM6 were therefore determined by PCR amplification (Kleppe *et al.*, 1971; Saiki *et al.*, 1988) of the DNA sequences between the genes. To ensure that the PCR conditions chosen were capable of yielding specific products of at least 3 kb in length, control reactions were performed with λHtM2 DNA. Since the location and orientation of the 6-1 $tRNA^{Tyr}$ gene on λHtM2 were known, this bacteriophage DNA served as an ideal control to test the PCR conditions. Several PCR amplifications were performed with primers chosen from a group of 12 oligonucleotides to find primer

Table 5. DNA fragments generated by restriction endonuclease digestion of λHtM4[a].

*Apa*I	*Bam*HI	*Bgl*II	*Eco*RI	*Hin*dIII	*Kpn*I
22.5	37.0 ♣♦♠	22.0 ♣♦♠	19.9	22.0 ♣♦♠	25.5 ♣♦♠
10.8 ♦[c]♠[d]	5.6	4.8	11.0	8.5	17.4
10.0	3.9	3.7	7.7 ♣♦♠	6.8	3.5
4.55 ♣[b]	1.5	2.7	3.2	5.9	1.5
		2.5	2.3	2.1	
		2.45	2.25	1.4	
		2x 2.0	1.5	0.95	
		1.4			
		1.0			
		0.90			
		0.80			
		0.70			

[a] The sizes of the restriction fragments are given in kb.

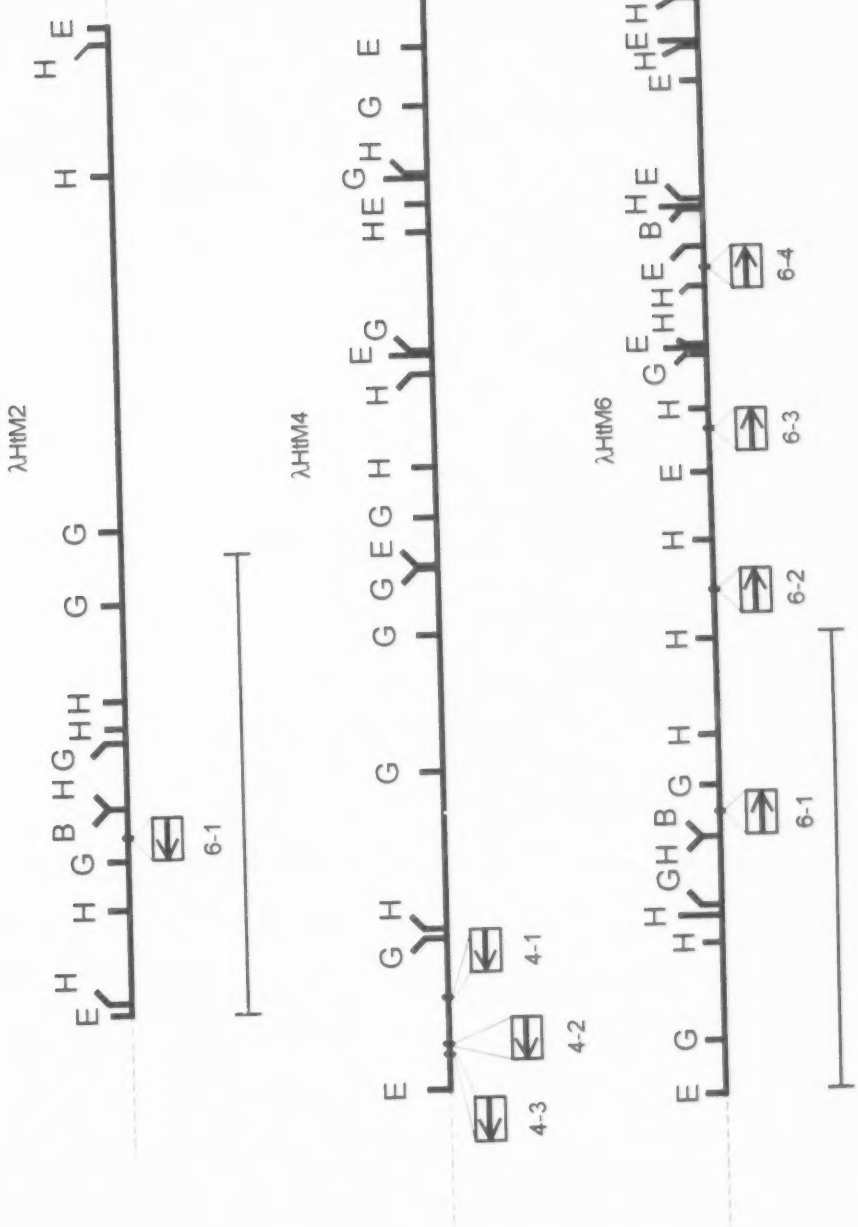
[b] The 4-1 $tRNA^{Tyr}$ gene-containing DNA fragments are indicated with a symbol (♣).

[c] The 4-2 $tRNA^{Tyr}$ gene-containing DNA fragments are indicated with a symbol (♦).

[d] The 4-3 $tRNA^{Ala}$ gene-containing DNA fragments are indicated with a symbol (♠).

Figure 16. Restriction endonuclease digestion maps of the three recombinant bacteriophage DNAs.

The λ Charon 4A phage vector sequences are shown as dashed lines, with the maps drawn from left to right. The human DNA inserts are represented by the darker solid lines. The restriction endonuclease digestion sites are indicated by letters with B for *Bam*HI, G for *Bgl*II, E for *Eco*RI and H for *Hin*dIII. The tRNA genes are represented by dots on the restriction maps; however, the orientations of the genes are shown below each map. The arrows enclosed in boxes represent tRNA genes, with the point of the arrow indicating the direction of transcription. The overlapping portions of λHtM2 and λHtM6 are indicated by solid lines under each respective restriction map.


λHtM2
λHtM4
λHtM6
6-1
4-1
4-2
4-3
6-2
6-3
6-4
1 kb

combinations that would yield PCR products extending from gene to gene. The 12 oligonucleotides, from which PCR primers were selected, consisted of 2 gene-specific primers for each $tRNA^{Tyr}$ gene on λHtM6, 2 general $tRNA^{Tyr}$ gene primers, and 2 λ-specific primers.

The primer combinations that generated PCR products reflected the $tRNA^{Tyr}$ gene orientations and the lengths of the PCR products confirmed the gene locations on λHtM6. However, the likelihood of PCR artifacts occurring in the reactions was high due to the very large degree of homology between the $tRNA^{Tyr}$ genes. Therefore, to distinguish authentic PCR products from PCR artifacts the amplified products were fractionated on agarose gels (Figure 17 Panels A and B), transferred to nylon membranes and hybridized sequentially with $tRNA^{Tyr}$ gene-specific probes. In most cases when the primer combinations gave rise to PCR products, the principal PCR product was found to hybridize with $tRNA^{Tyr}$ gene-specific probes. Although, in two instances (Figure 17 Panel A, lane 12; Figure 17 Panel B, lane 11) a minor 2.5 kb PCR product, rather than the predominant 4.5 kb PCR product, hybridized with the 6-3 and 6-4 $tRNA^{Tyr}$ gene-specific probes. The autoradiographs of these Southern transfers allowed the visualization of the PCR products that extended from gene to gene (data not shown). The conclusions from the PCR reactions and the hybridizations are summarized in Figure 18, illustrating the λ HtM6 $tRNA^{Tyr}$ gene locations and orientations.

3.3 DNA sequencing

While five of the six tyrosine tDNA sequences, as well as their immediate flanking sequences, had been determined by MacPherson (1988) one $tRNA^{Tyr}$ gene sequence remained to be determined. The $tRNA^{Tyr}$ gene that remained uncharacterized was named M2 and it was located on λHtM2. To characterize this $tRNA^{Tyr}$ gene, a 855 bp *Hin*dIII/*Bgl*II fragment known to contain this gene was cloned into pUC118 and then sequenced by the chain termination method (Figure 19). Analysis of this DNA sequence

Figure 17. Determination of the λHtM6 tRNA^{Tyr} gene orientations by PCR.

Panel A

A photograph of the ethidium bromide stained 1.0 % agarose gel on which samples of the PCR reactions were fractionated. Lane 1 contains λ DNA digested with restriction endonuclease *Bst*EII. Lanes 2 - 17 show the products from the amplification of λHtM6 DNA, while lanes 18 and 19 show the products from the amplification of λHtM2 DNA. The primers that were used for each PCR reaction are indicated in the table below, under each lane number. See Section 2.11 (Materials and Methods) for the sequence of each primer.

	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Tyrosine Primer DSP 1	X	X															X	
pM6128 Primer KLR 77			X	X														X
pM6 Primers DSP 19	X		X		X	X												
KLR 79		X		X			X	X										
pM612 Primers DSP 21					X		X		X	X					X			
KLR 80						X		X			X	X				X		
pM6IT Primers DSP 22									X		X		X					
KLR 78										X		X		X				
LEFT λ Primer DSP 5																	X	X
RIGHT λ Primer DSP 6													X	X	X	X		

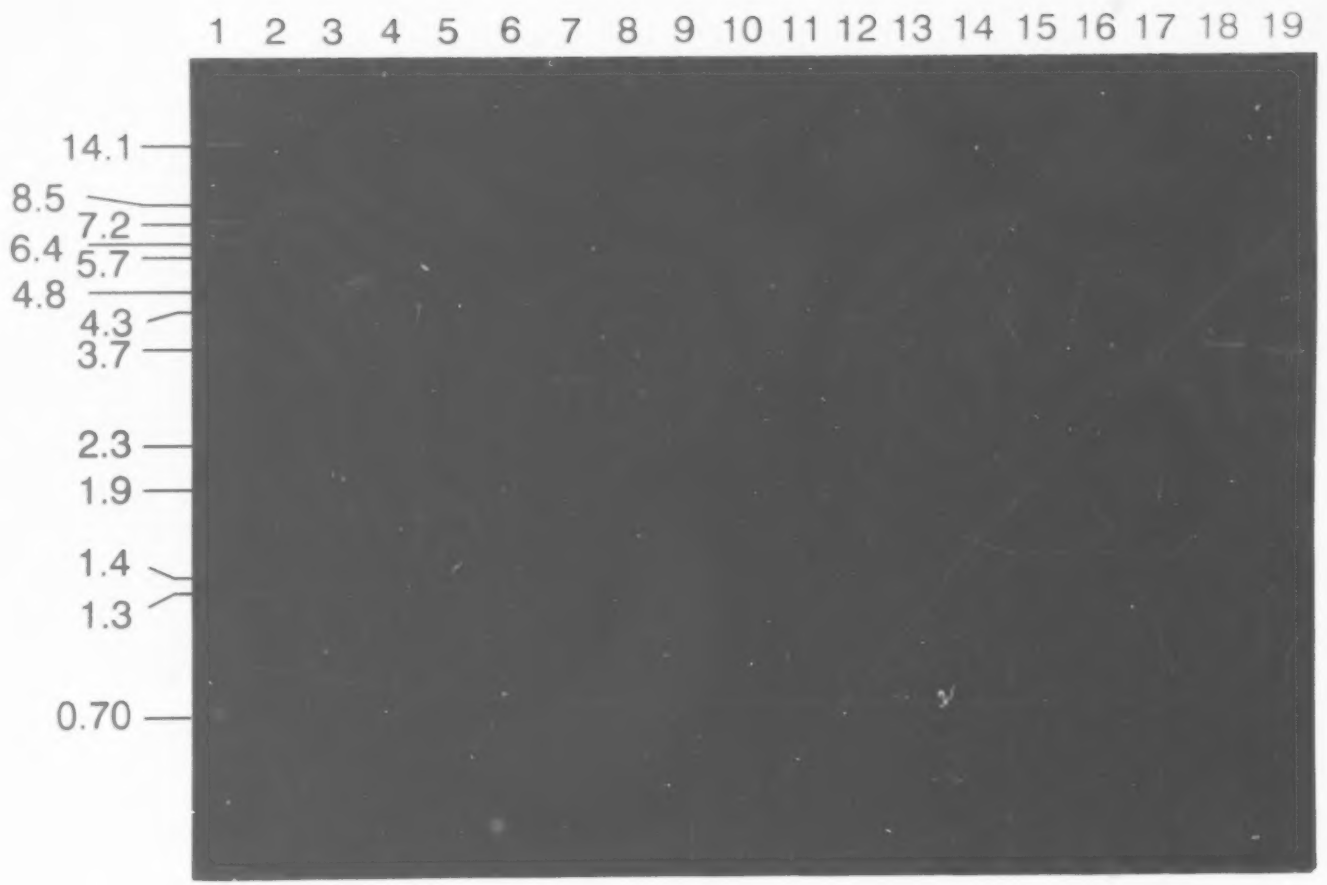

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
14.1
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.4
1.3
0.70

Figure 17. Determination of the λHtM6 tRNA^{Tyr} gene orientations by PCR.

Panel B

A photograph of the ethidium bromide stained 1.0 % agarose gel on which samples of the PCR reactions were fractionated. Lanes 1, λ DNA digested with restriction endonuclease *Bst*EII, and 16, λ DNA digested with restriction endonuclease *Hin*dIII, are DNA size markers. Lanes 2 - 15 show the products from the amplification of λHtM6 DNA. The primers that were used for each PCR reaction are indicated in the table below, under each lane number. See Section 2.11 for the sequence of each primer.

	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Tyrosine Primers DSP 1 (Tyr-R) DSP 2 (Tyr-L)	X	X	X	X	X	X	X	X	X	X	X	X	X	X
pM6128 Primer KLR 77			X						X					
pM6 Primer KLR 79					X						X			
pM612 Primer KLR 80				X						X				
pM6IT Primer KLR 78						X						X		
LEFT λ Primer DSP 5								X						X
RIGHT λ Primer DSP 6							X						X	

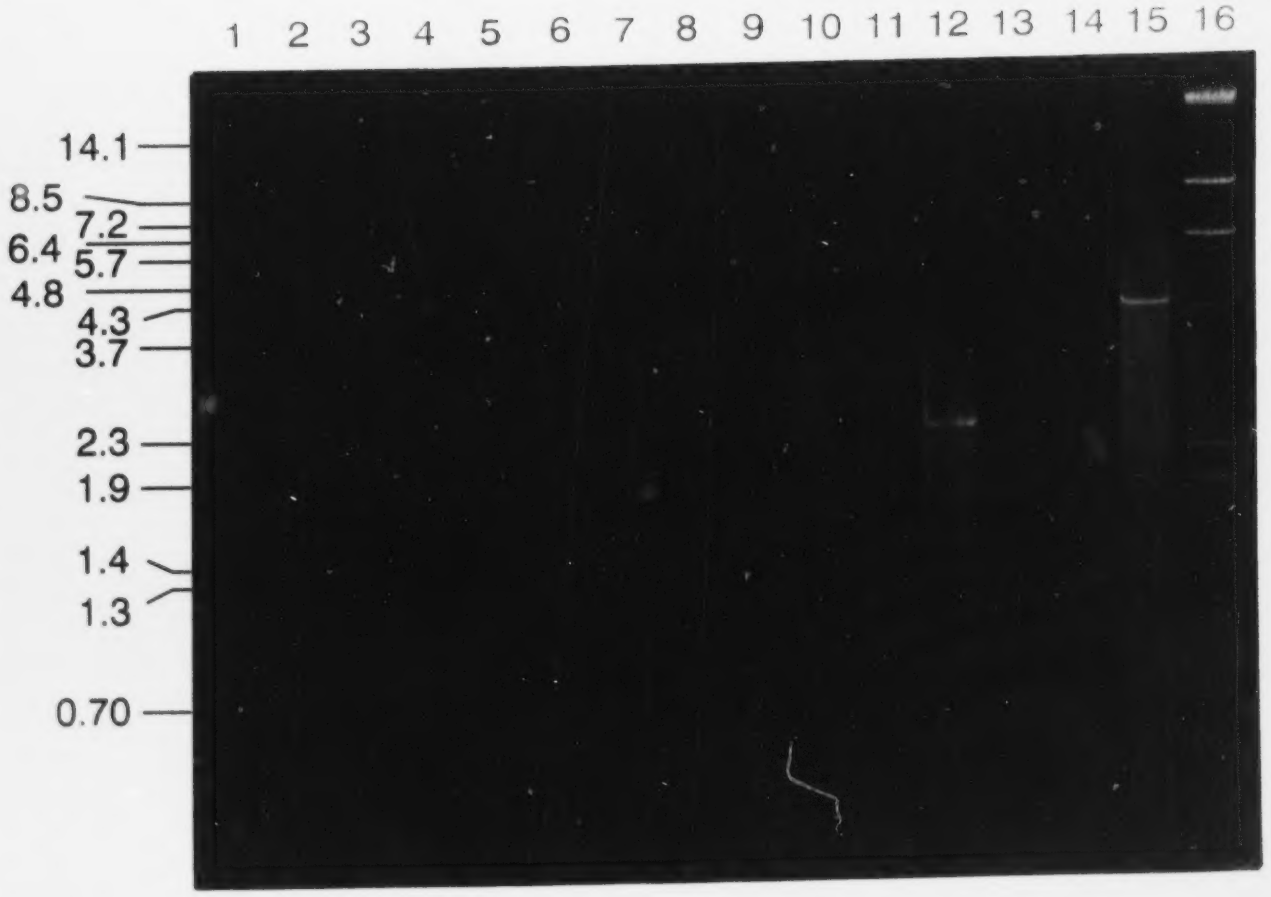

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
14.1
8.5
7.2
6.4
5.7
4.8
4.3
3.7
2.3
1.9
1.4
1.3
0.70

Figure 18. Schematic representation of the PCR products spanning from gene to gene on recombinant bacteriophage λHtM6.

The λ Charon 4A phage vector sequences are shown as dashed lines, with the maps drawn from left to right. The DNA insert is represented by the darker solid line. The restriction endonuclease digestion sites are indicated by letters with B for *Bam*HI, G for *Bgl*II, E for *Eco*RI and H for *Hin*dIII. The tRNA genes are represented by dots on the restriction maps, however, the orientations of the genes are shown below each map. The arrows enclosed in boxes represent tRNA genes, with the point of the arrow indicating the direction of transcription. The overlapping portion between λHtM2 and λHtM6 is indicated by solid line under the restriction map. The three most important PCR products are shown below the restriction map, along with the primers used to amplify these products. Note that the primers, represented by arrows, are not drawn the scale.

λHtM6

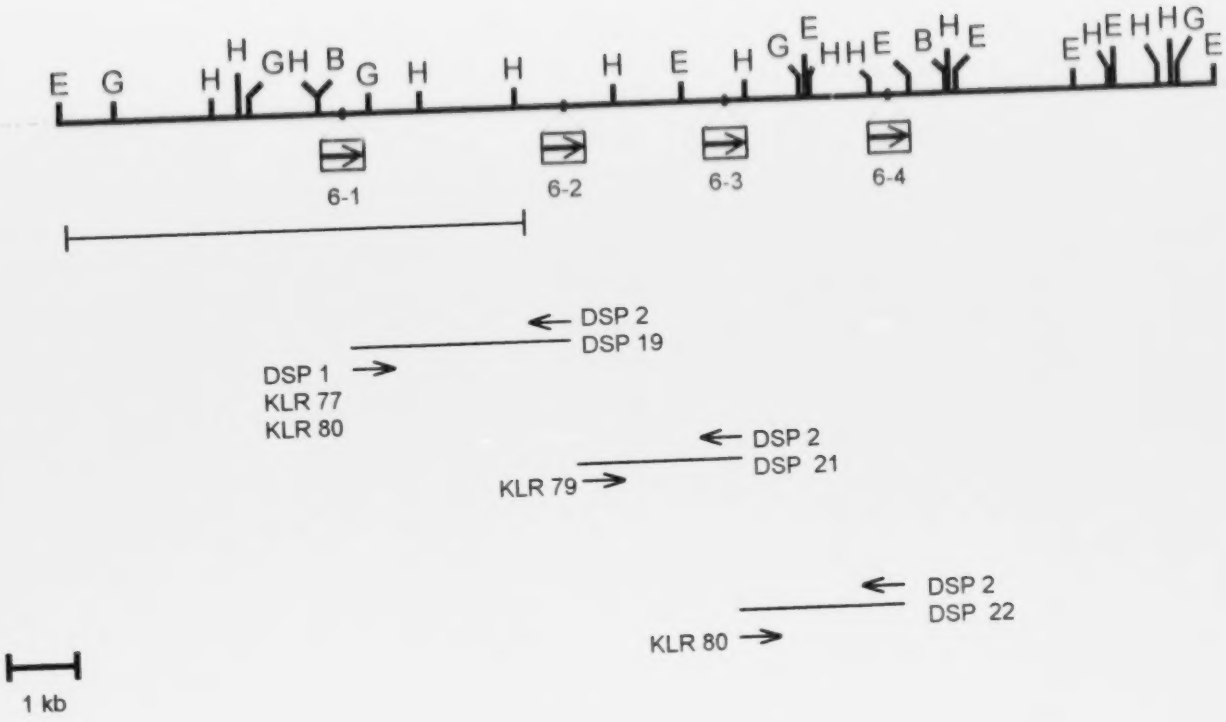

E G H H GH B G H H H E H G E HH E B H E E H E H H G E
6-1
6-2
6-3
6-4
DSP 2
DSP 19
DSP 1
KLR 77
KLR 80
DSP 2
DSP 21
KLR 79
DSP 2
DSP 22
KLR 80
1 kb

Figure 19. Nucleotide sequence of the $tRNA^{Tyr}$ gene within pM6128.

The nucleotide sequence of the 855 bp *Hin*dIII/*Bgl*II DNA fragment from λHtM2 is shown. The upper strand of sequence depicts the non-coding strand of DNA. The 6-1 $tRNA^{Tyr}$ gene which starts at position 395 is shown in bold, while the intervening sequence is underlined. A transcription termination signal of 4 T residues is found at position 501. The nucleotide sequence initially determined by MacPherson (1988) is indicated with dashed lines.

```
            10         20         30         40         50
             |          |          |          |          |
  1 AAGCTTTCCG AGTTCTTCTT TCCTCCTCCT GCAACCCCTG TCCCACAGGA
    TTCGAAAGGC TCAAGAAGAA AGGAGGAGGA CGTTGGGGAC AGGGTGTCCT

 51 TCCACCCATC CACCTAGCCC ATCCCTCTGA CCGAGCCCTC TCACCCTCTT
    AGGTGGGTAG GTGGATCGGG TAGGGAGACT GGCTCGGGAG AGTGGGAGAA

101 GTTTTCTTTC GCTGAGGGCT GTCATCCTCA CTTGTAAAAA CAGAGATGCA
    CAAAAGAAAG CGACTCCCGA CAGTAGGAGT GAACATTTTT GTCTCTACGT

151 CAGGTGGAGG AAGGCCACAG GCGAGAGCCT CCCGTCCTGG ATTGTGGCTA
    GTCCACCTCC TTCCGGTGTC CGCTCTCGGA GGGCAGGACC TAACACCGAT

201 TCAGCGCTCT GGGACGCGAG GAAACCACAC TCGGAGGATT TGCTCCACCC
    AGTCGCGAGA CCCTGCGCTC CTTTGGTGTG AGCCTCCTAA ACGAGGTGGG

251 TGAGAGGTGC GCGGTGGCAA CCAGCGCAAG GTTCTCTTCT AAGGCGGGTT
    ACTCTCCACG CGCCACCGTT GGTCGCGTTC CAAGAGAAGA TTCCGCCCAA

301 CCAATCAACT CTAAGTGTGT TGACTCCAGC GTTCCAAGGA CTTGGCTTCC
    GGTTAGTTGA GATTCACACA ACTGAGGTCG CAAGGTTCCT GAACCGAAGG

351 TCCATTTGCG GAAAGTCCAG TGATCCAGCT CTTGCAGCGT GCACCTTCG
    AGGTAAACGC CTTTCAGGTC ACTAGGTCGA GAACGTCGCA CGTGGGAAGC

401 ATAGCTCAGC TGGTAGAGCG GAGGACTGTA GATTGTACAG ACATTTGCGG
    TATCGAGTCG ACCATCTCGC CTCCTGACAT CTAACATGTC TGTAAACGCC

451 ACATCCTTAG GTCGCTGGTT CGATTCCGGC TCGAAGGAAG TGCCCGATGC
    TGTAGGAATC CAGCGACCAA GCTAAGGCCG AGCTTCCTTC ACGGGCTACG

501 TTTTGCATGC AATGCCACCT GGTGCCTGGT CAAACGCCCT GCAGCCTCCA
    AAAACGTACG TTACGGTGGA CCACGGACCA GTTTGCGGGA CGTCGGAGGT

551 ACTAGTATCC ACCCACACCC TCCCAGTCAA AACCCAGAGA AACCTTTCCT
    TGATCATAGG TGGGTGTGGG AGGGTCAGTT TTGGGTCTCT TTGGAAAGGA

601 GATCACCTGG TTTCCACACC TGTGCTGTGG CCAGGAAACA CGCCCGTAAG
    CTAGTGGACC AAAGGTGTGG ACACGACACC GGTCCTTTGT GCGGGCATTC

651 CCCACTCATT CTTCCCACAG TCCAGGGACA GGTACTCTTC AACGCAAAGG
    GGGTGAGTAA GAAGGGTGTC AGGTCCCTGT CCATGAGAAG TTGCGTTTCC

701 GCCCCTTGCT TCTGCTTTGT GACCCAGTCA GCCCCTCTCC CCAACCCCCT
    CGGGGAACGA AGACGAAACA CTGGGTCAGT CGGGGAGAGG GGTTGGGGGA

751 TCGCACCCCC ACCATCCAGA GCTCTTTCAC TTTTATCCAC AGACTGCTCT
    AGCGTGGGGG TGGTAGGTCT CGAGAAAGTG AAAATAGGTG TCTGACGAGA

801 GGGTGCTGAG GGCCATGGAC TCTAGCAACA TTGTGTGCTC ACCATTGATA
    CCCACGACTC CCGGTACCTG AGATCGTTGT AACACACGAG TGGTAACTAT

851 GATCT
    CTAGA
```

showed that the $tRNA^{Tyr}$ gene and its surrounding flanking sequences were identical to the 6-1 $tRNA^{Tyr}$ gene on λHtM6. This was the observation which led to further experiments which have since shown that λHtM2 and λHtM6 are overlapping bacteriophage clones. The series of clones constructed to sequence this 855 bp DNA fragment were later utilized for *in vitro* transcription experiments.

An additional project undertaken was the sequencing of a 2.4 kb *Hin*dIII/*Eco*RI fragment of λHtM4 which was thought to contain an additional $tRNA^{Tyr}$ gene, based on the hybridization of $tRNA^{Tyr}$ gene-specific probes to two distinct bands on a Southern transfer of doubly digested pJM4 DNA. This fragment had been cloned into pAT153 (to give pJM4 by MacPherson (1988). The decision was made to subclone this fragment into pUC118 to facilitate DNA sequencing in both orientations. The multiple cloning site of pUC118 was utilized for the generation of overlapping clones by the exonuclease III unidirectional deletion method (Henikoff, 1987). The resulting deletion clones were selected on the basis of size and the presence of a universal primer binding site, with suitable clones used for the production of single-stranded plasmid DNA to be used for sequencing with the Klenow fragment of *E. coli* DNA polymerase I. Double-stranded sequencing with Taq DNA polymerase was also performed. In places where deletion clones did not overlap, specific oligonucleotide primers were designed and synthesized to extend the sequence. In addition to the deletion clones generated, restriction fragments were subcloned into pUC118 to aid in the sequence determination and to serve as templates for *in vitro* transcription assays. This study identified two new tRNA genes, a $tRNA^{Tyr}$ gene and a $tRNA^{Ala}$ gene (Figure 20), that have been shown in the next section to be transcriptionally active.

Further sequencing of the tRNA gene-containing clones was undertaken using the Applied Biosystems Inc. automated DNA sequencer. The nucleotide sequence of the 1.4 kb λHtM6 *Hin*dIII fragment that carries the 6-2 $tRNA^{Tyr}$ gene was determined by automated sequencing utilizing a series of custom oligonucleotide primers (Figure 21).

Figure 20. Nucleotide sequence of the tRNA genes within pJM4.

The nucleotide sequence of the 2455 bp *Hin*dIII/*Eco*RI DNA fragment from λHtM4 is shown. The upper strand of the sequence depicts the non-coding strand of DNA. The tRNA genes are shown in bold and the intervening sequences are underlined, $tRNA^{Tyr}$ 4-1 at position 1117, $tRNA^{Tyr}$ 4-2 at position 1738, and $tRNA^{Ala}$ 4-3 at position 1940. Termination signals (4 or more T residues) are found at positions 1221, 1849, 2014, and 2036. The nucleotide sequence initially determined by MacPherson (1988) is indicated with dashed lines.

```
            10          20          30          40          50
            |           |           |           |           |
  1 AAGCTTTTAC TTTTGGGAAC TGCTATCTTA CAGGGGAGAT TTAAGAAATA
    TTCGAAAATG AAAACCCTTG ACGATAGAAT GTCCCCTCTA AATTCTTTAT

 51 ATTCCAATCT CCCTTCCACA AATATTACTA TAATAACATT AAAATAGAAA
    TAAGGTTAGA GGGAAGGTGT TTATAATGAT ATTATTGTAA TTTTATCTTT

101 CTCAGTTTGG CCAAACCAAC ATGATGCCTT CTTGGTTAAA AGATCTCTAA
    GAGTCAAACC GGTTTGGTTG TACTACGGAA GAACCAATTT TCTAGAGATT

151 GGGCAAAACC CAGGTCGTTG GATGGTTTTT ACGCTACGCT AACAACTGAG
    CCCGTTTTGG GTCCAGCAAC CTACCAAAAA TGCGATGCGA TTGTTGACTC

201 TGGGAATATG GCAGCAAACC TTAACAATTT ATAAACGCTA TGACCTTACA
    ACCCTTATAC CGTCGTTTGG AATTGTTAAA TATTTGCGAT ACTGGAATGT

251 AAAATAGGCC GACGAAGCAG GATTATAAGC CTATCTGGGA TTGAATAACT
    TTTTATCCGG CTGCTTCGTC CTAATATTCG GATAGACCCT AACTTATTGA

301 TTTTAAAATT AAACATAAAA TAGGAACCAG ATCAAAAAGA CATTCTATGT
    AAAATTTTAA TTTGTATTTT ATCCTTGGTC TAGTTTTTCT GTAAGATACA

351 GATCTTCTTC CCTCTCTTTG CTTAGCCGGG GGTTGGTGAG GGGACTTTCT
    CTAGAAGAAG GGAGAGAAAC GAATCGGCCC CCAACCACTC CCCTGAAAGA

401 CTAGGAACTT AGTGACTCTG ATGTCTTAGT AGATGCGGTG GAACCAGCTC
    GATCCTTGAA TCACTGAGAC TACAGAATCA TCTACGCCAC CTTGGTCGAG

451 TGTCTACAGA ACCCCGGGCT TCAGTGGCGT CTTCCTAACC CGGCTTGCCT
    ACAGATGTCT TGGGGCCCGA AGTCACCGCA GAAGGATTGG GCCGAACGGA

501 GCCGGGGGCG TTCCGAGACC CTCGGGGCCT TCCCTTCACC CCGCGGGAGT
    CGGCCCCCGC AAGGCTCTGG GAGCCCCGGA AGGGAAGTGG GGCGCCCTCA

551 TGGACCGGTG GCGCTGGTAA GGCCTCCCGG GCTCAAAGTG CAACGGACAC
    ACCTGGCCAC CGCGACCATT CCGGAGGGCC CGAGTTTCAC GTTGCCTGTG

601 TGCAGAAATC CAAACTGCTG GCATTCGCGG TTTGGGGACG CCAGAGGAGG
    ACGTCTTTAG GTTTGACGAC CGTAAGCGCC AAACCCCTGC GGTCTCCTCC

651 TAATGATTTC TGGTTTGTTA ACCTCAAGTG ACAATAATGC CGAGCCAGGC
    ATTACTAAAG ACCAAACAAT TGGAGTTCAC TGTTATTACG GCTCGGTCCG

701 AGGAGCTGGA CCTACAATCT TCTGATGCGT GGTCAGACAC GTTATCCCTT
    TCCTCGACCT GGATGTTAGA AGACTACGCA CCAGTCTGTG CAATAGGGAA

751 GCGCCACTGG CCTACCACGC TACTCCTTCA GTCGCCGTTG GATTACTGTG
    CGCGGTGACC GGATGGTGCG ATGAGGAAGT CAGCGGCAAC CTAATGACAC

801 TGTTGAGAAC ACACTCGGCA ACCACTTTAA AGGACAACGC AGGCTGGTAA
    ACAACTCTTG TGTGAGCCGT TGGTGAAATT TCCTGTTGCG TCCGACCATT

851 AGGAAAAGTA CGACAAGGGG GGGCGGTGGA ATCGCAGGGT CTTGGCATCG
    TCCTTTTCAT GCTGTTCCCC CCCGCCACCT TAGCGTCCCA GAACCGTAGC

901 CGGACCCCAG ACACCTGGGT TGAGGGCCTT TCCCGGGTCA GTCAGGCTAG
    GCCTGGGGTC TGTGGACCCA ACTCCCGGAA AGGGCCCAGT CAGTCCGATC
```

```
                10          20          30          40          50
                |           |           |           |           |
 951 CGAGCCGGAG CGTTCTGTCT TTCTGCGCAC GCGTAGAGCA CACAGGCCGG
     GCTCGGCCTC GCAAGACAGA AAGACGCGTG CGCATCTCGT GTGTCCGGCC

1001 CTCTGGGGCT CTGCGCTCCT CGGATTACGC ATGCTCAGTG CAATCTTCGG
     GAGACCCCGA GACGCGAGGA GCCTAATGCG TACGAGTCAC GTTAGAAGCC

1051 TTGCCTGGAC TAGCGCTCCG GTTTTTCTGT GCTGAACCTC AGGGGACGCC
     AACGGACCTG ATCGCGAGGC CAAAAAGACA CGACTTGGAG TCCCCTGCGG

1101 GACACACGTA CACGTCCCTT CGATAGCTCA GCTGGTAGAG CGGAGGACTG
     CTGTGTGCAT GTGCAGGGAA GCTATCGAGT CGACCATCTC GCCTCCTGAC

1151 TAGCTACTTC CTCAGCAGGA GACATCCTTA GGTCGCTGGT TCGATTCCGG
     ATCGATGAAG GAGTCGTCCT CTGTAGGAAT CCAGCGACCA AGCTAAGGCC

1201 CTCGAAGGAG ACAAGTGCGG TTTTTTTTCT CCAGCTCCCG ATGACTTATG
     GAGCTTCCTC TGTTCACGCC AAAAAAAAGA GGTCGAGGGC TACTGAATAC

1251 GCACTTTCCT TGGGTGCCTT CAGTGACACA TTGCATTCCA ACGAGCAGTT
     CGTGAAAGGA ACCCACGGAA GTCACTGTGT AACGTAAGGT TGCTCGTCAA

1301 TGAAAGTCTA GCGCTTTCTC CCCATTTTGG GCCTCCCAGC CTGCACGGTA
     ACTTTCAGAT CGCGAAAGAG GGGTAAAACC CGGAGGGTCG GACGTGCCAT

1351 ATTCTTTTTA GCCATTCGCC CTGCGGGAAC GTGTCCGGGC AGGTTCCCAG
     TAAGAAAAAT CGGTAAGCGG GACGCCCTTG CACAGGCCCG TCCAAGGGTC

1401 CGCAGTGTGG GTCTGCGCTT GGCCGAGCGA CTGCCGGGTC ACGACTTCTG
     GCGTCACACC CAGACGCGAA CCGGCTCGCT GACGGCCCAG TGCTGAAGAC

1451 CGTCTTCTTA ACCCGTCTTT GGCATTGCCC GGGCCCCGAG TCACACAGGA
     GCAGAAGAAT TGGGCAGAAA CCGTAACGGG CCCGGGGCTC AGTGTGTCCT

1501 GGCAGCGCCG GCTCCAGGGG GCCAGGCGGG GACCTTCTCC TCAGAGCCCC
     CCGTCGCGGC CGAGGTCCCC CGGTCCGCCC CTGGAAGAGG AGTCTCGGGG

1551 GGGCAGCTTC TGCGACCCGA GGGCTCGCAA CGGCTGCCGT GAGGAGGTGG
     CCCGTCGAAG ACGCTGGGCT CCCGAGCGTT GCCGACGGCA CTCCTCCACC

1601 GGGGTCCGCG GGAAGAGGTA TCTGGCGCTC CCGGAACCTG GGAATCAGAA
     CCCCAGGCGC CCTTCTCCAT AGACCGCGAG GGCCTTGGAC CCTTAGTCTT

1651 AGAGAGAACA CAATACTAAA AACACGAAGC CTAAAAATGA CACAATGTTA
     TCTCTCTTGT GTTATGATTT TTGTGCTTCG GATTTTTACT GTGTTACAAT

1701 TGGAGACAAG GCGGCACCCG GGAAGCTGTG CCCGCTCCCT TCGATAGCTC
     ACCTCTGTTC CGCCGTGGGC CCTTCGACAC GGGCGAGGGA AGCTATCGAG

1751 AGCTGGTAGA GCGGAGGACT GTAGGCGCGC GCCCGTGGCC ATCCTTAGGT
     TCGACCATCT CGCCTCCTGA CATCCGCGCG CGGGCACCGG TAGGAATCCA

1801 CGCTGGTTCG ATTCCGGCTC GAAGGAGAGA CACCCCCCCC CCCCATTATT
     GCGACCAAGC TAAGGCCGAG CTTCCTCTCT GTGGGGGGGG GGGGTAATAA

1851 TTGTTGCTTT GAACCAAAAA AGTCTGTCTT CAGCGCTCAA TGTTCTGACC
     AACAACGAAA CTTGGTTTTT TCAGACAGAA GTCGCGAGTT ACAAGACTGG
```

```
                10          20          30          40          50
                |           |           |           |           |
1901 CTTCTCTAAA GGAACAGATA ATAAGCCGTG CCCAGCCGTG GGGGATTAGC
     GAAGAGATTT CCTTGTCTAT TATTCGGCAC GGGTCGGCAC CCCCTAATCG

1951 TCAAATGGTA GAGCGCTCGC TTAGCATGCG AGAGGTAGCG GGATCGATGC
     AGTTTACCAT CTCGCGAGCG AATCGTACGC TCTCCATCGC CCTAGCTACG

2001 CCGCATCCTC CAGTTTTCCT TCCTGTCCCG TACGGTTTTT CTTTCGATTC
     GGCGTAGGAG GTCAAAAGGA AGGACAGGGC ATGCCAAAAA GAAAGCTAAG

2051 TCAGCCCAAA CTAGAGCTGA AAAGTCAGAC GAAGTCAGGT GAAGAGTAGG
     AGTCGGGTTT GATCTCGACT TTTCAGTCTG CTTCAGTCCA CTTCTCATCC

2101 GCGAGCTCCA GCTTACCACT CTAAAACTTC CCAGACAATG AGTGGTGGGC
     CGCTCGAGGT CGAATGGTGA GATTTTGAAG GGTCTGTTAC TCACCACCCG

2151 GCTCAGCAGT TTATCCTTCT AGCTTTAAAT TTTTAGCCCC ATTTAATTGG
     CGAGTCGTCA AATAGGAAGA TCGAAATTTA AAAATCGGGG TAAATTAACC

2201 GGGGAAATTC ACGAAACTGG TTATTTTTGC TTCAAAAATG GCGACAGATT
     CCCCTTTAAG TGCTTTGACC AATAAAAACG AAGTTTTTAC CGCTGTCTAA

2251 GCCGTCACAT GTATTATCAC TCAGAATCCT TTATGATTTG TGATAAGATG
     CGGCAGTGTA CATAATAGTG AGTCTTAGGA AATACTAAAC ACTATTCTAC

2301 TCTGCATTTC CAGGGACTCA TCTGAGGCTA AGCTGCCCAT AGTTCGGGGA
     AGACGTAAAG GTCCCTGAGT AGACTCCGAT TCGACGGGTA TCAAGCCCCT

2351 GACCCACAGG GAAAACAAAA CAAGAAACAG AGAACTTGGA AACGGACGCT
     CTGGGTGTCC CTTTTGTTTT GTTCTTTGTC TCTTGAACCT TTGCCTGCGA

2401 GATTACTTTG AACGTTTGCT CAACCGAGGA AGCAGGAACT GTTCGGCATG
     CTAATGAAAC TTGCAAACGA GTTGGCTCCT TCGTCCTTGA CAAGCCGTAC

2451 AATTC
     TTAAG
```

Figure 21. Nucleotide sequence of the $tRNA^{Tyr}$ gene within pM6.

The nucleotide sequence of the 1390 bp *Hin*dIII DNA fragment from λHtM6 is shown. The upper strand of sequence depicts the non-coding strand of DNA. The 6-2 $tRNA^{Tyr}$ gene which starts at position 998 is shown in bold, while the intervening sequence is underlined. A transcription termination signal of 4 T residues is found at position 1104. The coding strand of a putative Alu sequence, located between positions 89 to 386, is indicated by underlining, while the 4 bp direct repeats are indicated by double underlining. The nucleotide sequence initially determined by MacPherson (1988) is indicated with dashed lines.

```
          10         20         30         40         50
           |          |          |          |          |
    1 AAGCTTTTAT GCTGGCCTTT GGGGTTAGAG GTAATGGGTA TCACATGGTG
      TTCGAAAATA CGACCGGAAA CCCCAATCTC CATTACCCAT AGTGTACCAC

   51 ATCTGCGAGC CCCTGGCCCT ACTCTCTCCC CACATATCTT TTTTTTTTTT
      TAGACGCTCG GGGACCGGGA TGAGAGAGGG GTGTATAGAA AAAAAAAAAA

  101 TTTTTAAATT GTCTCTGTCG CCCAGGCTGG AGTGCAGTGG CAAGATCACA
      AAAAATTTAA CAGAGACAGC GGGTCCGACC TCACGTCACC GTTCTAGTGT

  151 GCTTACTGTA GCCTCGAACA CCCGGGCTCA GATGATCCTC CCACCTCAGC
      CGAATGACAT CGGAGCTTGT GGGCCCGAGT CTACTAGGAG GGTGGAGTCG

  201 CTGCTGAGTG GCCAGGACCA CAGGTGCAGG CACCACACCC GGATACTTTT
      GACGACTCAC CGGTCCTGGT GTCCACGTCC GTGGTGTGGG CCTATGAAAA

  251 TAGAAGTTTT TCTGTAGAGA TGGCTTCTCC CTATGTTGCC CAGGCTGATC
      ATCTTCAAAA AGACATCTCT ACCGAAGAGG GATACAACGG GTCCGACTAG

  301 TCGAACTCCT GCGTCAAGCG CCCCTTTCGC CTCGGCCCGC TAAATTGTTG
      AGCTTGAGGA CGCAGTTCGC GGGGAAAGCG GAGCCGGGCG ATTTAACAAC

  351 GAATTGCGGT GCGAGCCACC ATACCTGGCC TCCACCTATC CTCCTGCATT
      CTTAACGCCA CGCTCGGTGG TATGGACCGG AGGTGGATAG GAGGACGTAA

  401 TCCTCCCTCT CTTCCCATTA TGCCTCAATA CTCCAAAAAG TGAGCATAGG
      AGGAGGGAGA GAAGGGTAAT ACGGAGTTAT GAGGTTTTTC ACTCGTATCC

  451 ACACTGGGTA GAAGGGCCGC GCACATCGAG AGGAGTGTGT TTGAGGTGGT
      TGTGACCCAT CTTCCCGGCG CGTGTAGCTC TCCTCACACA AACTCCACCA

  501 GGGAAGTAGA GGACAGGCTG TTAGGGCAGT GCCCCCTTAT GGTCTTCCAT
      CCCTTCATCT CCTGTCCGAC AATCCCGTCA CGGGGGAATA CCAGAAGGTA

  551 CAGACCCTGA CGCTAGGCTG GGGTTGGAAG CTGCTTACAC CACGCCCATG
      GTCTGGGACT GCGATCCGAC CCCAACCTTC GACGAATGTG GTGCGGGTAC

  601 CTGGTTCTCC TCTTTTCCTC CTCCAGTGTC CTCTCCTCCA CTGGACCCAC
      GACCAAGAGG AGAAAAGGAG GAGGTCACAG GAGAGGAGGT GACCTGGGTG

  651 CCATCAGTCT CACCAAGCCC TCTGCCCTCG CCGTCTTACC TCATTTTCCC
      GGTAGTCAGA GTGGTTCGGG AGACGGGAGC GGCAGAATGG AGTAAAAGGG

  701 TCTGCCACTA AGGGCTGTCA TCTTCACAAG CAGAAAGAGC GATGCAGAGG
      AGACGGTGAT TCCCGACAGT AGAAGTGTTC GTCTTTCTCG CTACGTCTCC

  751 GAGAGGAAGG CCACAGGTGA GAGCTCCTCC TCGTGGATCG TGGCTATCGG
      CTCTCCTTCC GGTGTCCACT CTCGAGGAGG AGCACCTAGC ACCGATAGCC

  801 CGCCCCAGAG ACGCCAGGCA CCCCCCCGGG CCCAGAGGAT TTGCTCTGTC
      GCGGGGTCTC TGCGGTCCGT GGGGGGGCCC GGGTCTCCTA AACGAGACAG

  851 CGGAAAGGGG CGCAGTGGAG ACCGGCGCAG AGTTCTCTTC CAAGTCGGGT
      GCCTTTCCCC GCGTCACCTC TGGCCGCGTC TCAAGAGAAG GTTCAGCCCA

  901 TCCAATTAAC TCAACGAGTA TTGGATCTCC GGTGGTCCAG GGACTTGGCT
      AGGTTAATTG AGTTGCTCAT AACCTAGAGG CCACCAGGTC CCTGAACCGA
```

```
                10          20          30          40          50
                |           |           |           |           |
 951 TCCTCCATTT GCAGAAAGTC CAGTGACCCA GCCTTAACAG TGTGCATCCT
     AGGAGGTAAA CGTCTTTCAG GTCACTGGGT CGGAATTGTC ACACGTAGGA

1001 TCGATAGCTC AGCTGGTAGA GCGGAGGACT GTAGACTGCG GAAACGTTTG
     AGCTATCGAG TCGACCATCT CGCCTCCTGA CATCTGACGC CTTTGCAAAC

1051 TGGACATCCT TAGGTCGCTG GTTCAATTCC GGCTCGAAGG AAGCGCCTGA
     ACCTGTAGGA ATCCAGCGAC CAAGTTAAGG CCGAGCTTCC TTCGCGGACT

1101 CTCTTTTGCG CACAATGCTG CCTGGCTGCA CCTGTTCCTC GTCAAAGACC
     GAGAAAACGC GTGTTACGAC GGACCGACGT GGACAAGGAG CAGTTTCTGG

1151 TTGCAGCCTT CCAGTCATAA CTACACTTTC CCCAGGAAAA CCCAGCAAAA
     AACGTCGGAA GGTCAGTATT GATGTGAAAG GGGTCCTTTT GGGTCGTTTT

1201 TCCTGCCTTT CCTGATCACA GGCCTGGGAG CCTATTCATT CTTCCCGAAT
     AGGACGGAAA GGACTAGTGT CCGGACCCTC GGATAAGTAA GAAGGGCTTA

1251 CCTCATAGTC TTCAGCTCAA AACAGTGCCC CTTGCCTTGT GACCCAGCCA
     GGAGTATCAG AAGTCGAGTT TTGTCACGGG GAACGGAACA CTGGGTCGGT

1301 GACCCCAATT CGTGCCCCCA CCATCCTACG CCTTTCCACT TGTATTTACA
     CTGGGGTTAA GCACGGGGGT GGTAGGATGC GGAAAGGTGA ACATAAATGT

1351 AACTGCCCAC CCATCCCCCT GTCCCAGACC CGTCAAGCTT
     TTGACGGGTG GGTAGGGGGA CAGGGTCTGG GCAGTTCGAA
```

The nucleotide sequence of the 2.0 kb λHtM6 *Hin*dIII fragment that carries the 6-3 $tRNA^{Tyr}$ gene has also been determined in a similar fashion (Figure 22). Additional sequences surrounding the 6-4 $tRNA^{Tyr}$ gene have also been sequenced (Figure 23). The flanking sequences of the $tRNA^{Tyr}$ genes from λHtM6 were found to have extensive regions of homology. An alignment of the four gene-containing sequences reveals the extent of this homology (Figure 24).

A series of amplified tDNA sequences, cloned into pBS, have also been sequenced in both orientations. The tDNA sequences that were successfully amplified and cloned were from the 4-1, 6-1, 6-2, 6-3 and 6-4 $tRNA^{Tyr}$ genes (Figure 25). However, due to two degenerate positions on one of the oligonucleotide primers, mutations were introduced into some of the tDNA sequences. These pBS clones were constructed to serve as DNA templates for *in vitro* transcription experiments described in the next section.

3.4 *In vitro* transcription analysis of cloned human tRNA genes in mammalian cell extracts

To accomplish the major goal of this study, which was the identification of extragenic sequences that modulate human $tRNA^{Tyr}$ gene expression, a collection of $tRNA^{Tyr}$ gene-containing plasmid clones was used to direct the synthesis of pre-$tRNA^{Tyr}$ transcripts in mammalian cell extracts. Restriction maps of these recombinant plasmids show the positions of the $tRNA^{Tyr}$ genes and the amounts of flanking sequences surrounding the genes (Figures 26 - 30). Two human cell lines, HeLa and 293, were used for the preparation of cell extracts. Since relatively large amounts of cells were required (approx. 4 - 5 g) for these preparations, the cell lines were grown in suspension culture to reduce the labor involved with cell propagation. In order to obtain the most transcriptionally active cell extracts two whole cell extract procedures, one described by Manley *et al.* (1980) and the other described by Weil *et al.* (1979), were compared. The

Figure 22. Nucleotide sequence of the $tRNA^{Tyr}$ gene within pM612.

The nucleotide sequence of the 1971 bp *Hin*dIII DNA fragment from λHtM6 is shown. The upper strand of sequence depicts the non-coding strand of DNA. The 6-3 $tRNA^{Tyr}$ gene which starts at position 1561 is shown in bold, while the intervening sequence is underlined. A transcription termination signal of 4 T residues is found at position 1667. The nucleotide sequence initially determined by MacPherson (1988) is indicated with dashed lines.

```
          10         20         30         40         50
          |          |          |          |          |
  1 AAGCTTCCCG ATGTTTGATG TAAAGATGCA ACCTATCAGA GAGTACTCCA
    TTCGAAGGGC TACAAACTAC ATTTCTACGT TGGATAGTCT CTCATGAGGT

 51 AACTGAATGG CCCAGGAAAG CATGGCCTTC TGAAGCCTGC TTAGGACTGG
    TTGACTTACC GGGTCCTTTC GTACCGGAAG ACTTCGGACG AATCCTGACC

101 CTTGCCCCAT CTACTACCTG CTGGGTCCAC ATGAACTGTT TAATTGTGCC
    GAACGGGGTA GATGATGGAC GACCCAGGTG TACTTGACAA ATTAACACGG

151 TCTCAAACTG GATACTGCAC ATATTACTGC ACATATCTTT TTCACATGGA
    AGAGTTTGAC CTATGACGTG TATAATGACG TGTATAGAAA AAGTGTACCT

201 AAGCAGCTCC TGGTATCCCG CCACCGCCTA TTCTCCCCCA CGTCACCCCG
    TTCGTCGAGG ACCATAGGGC GGTGGCGGAT AAGAGGGGGT GCAGTGGGGC

251 ACTGTGTCAA CCTTTCTTCT TTGGTGTCAC CAGTGCTCTG GGATGCTTCT
    TGACACAGTT GGAAAGAAGA AACCACAGTG GTCACGAGAC CCTACGAAGA

301 ATGGCTCTGG AGGCACAGAG AGACCCCAGC TCCAATGACA CCAAAGGCAA
    TACCGAGACC TCCGTGTCTC TCTGGGGTCG AGGTTACTGT GGTTTCCGTT

351 AGACCAGCTA ACAAAGAGGG ACCAAAGGTA GCACCTCAGG CCTTCATTGG
    TCTGGTCGAT TGTTTCTCCC TGGTTTCCAT CGTGGAGTCC GGAAGTAACC

401 ATATATTCCT GATGGGGCGT GGAGTCACCA GAGCCCTTGG AACCTTTGCT
    TATATAAGGA CTACCCCGCA CCTCAGTGGT CTCGGGAACC TTGGAAACGA

451 CAGTGCTTTG GGGAAAAACC AGAGGTGAGC CAACAAATGG GTTTGGTGGC
    GTCACGAAAC CCCTTTTTGG TCTCCACTCG GTTGTTTACC CAAACCACCG

501 TGGCACAAGT GAAGGTGAGC CCAGGTGCCC ACTCTTCCCA GCTGTGCCAT
    ACCGTGTTCA CTTCCACTCG GGTCCACGGG TGAGAAGGGT CGACACGGTA

551 GGCAGAGAGT AGCAGGATGT CTGTGAGGAT CTGATCCTCA CTCTCGGAGA
    CCGTCTCTCA TCGTCCTACA GACACTCCTA GACTAGGAGT GAGAGCCTCT

601 TCCACACCCA CTTGCCATAA GACAGGAAGT GGACATAAAC TCAATGGAGG
    AGGTGTGGGT GAACGGTATT CTGTCCTTCA CCTGTATTTG AGTTACCTCC

651 CTAGGTCCCG TGGGTGTGGC TTCCTGTCCT CCTCTAGGTG TTCTGCTGCA
    GATCCAGGGC ACCCACACCG AAGGACAGGA GGAGATCCAC AAGACGACGT

701 GAGAGGGTGC CTCAGTGGTC TCGGAGTGGT GGGCACACAC CAGATGGGAT
    CTCTCCCACG GAGTCACCAG AGCCTCACCA CCCGTGTGTG GTCTACCCTA

751 CTGAAACTTT TGTCAAAGAT GTGGGTTCAG GATAAGGAGT CCAAAGCTCA
    GACTTTGAAA ACAGTTTCTA CACCCAAGTC CTATTCCTCA GGTTTCGAGT

801 CACTTCTGTC CTCTACCTGG CTTCTTGTTT TTCAAAGAAA CCCACAGAAC
    GTGAAGACAG GAGATGGACC GAAGAACAAA AAGTTTCTTT GGGTGTCTTG
```

```
                10         20         30         40         50
                 |          |          |          |          |
851 TTTGCCCTAC TTCAGAAATC AGCAGCTTCT GATTGGAATT CTCAGCCTGT
    AAACGGGATG AAGTCTTTAG TCGTCGAAGA CTAACCTTAA GAGTCGGACA

901 TTGCAGAAGA GGGTATCTCA CATGTGCCTC TGCAAGCCTG GGAGAGATTT
    AACGTCTTCT CCCATAGAGT GTACACGGAG ACGTTCGGAC CCTCTCTAAA

951 CCTCACCTCA CTTCCCAGTG CACCTCAATG CACCAAAATG TGAGCATAGA
    GGAGTGGAGT GAAGGGTCAC GTGGAGTTAC GTGGTTTTAC ACTCGTATCT

1001 CTACTATGAG GAGAAGGGCG GGCCACCTGG GGAGGAGGCC CCTGTGTGTG
     GATGATACTC CTCTTCCCGC CCGGTGGACC CCTCCTCCGG GGACACACAC

1051 TGGTAGGGGA GGGAGTAGAG AGGCCAGACT ATTATGGAAG CGCCCATTAG
     ACCATCCCCT CCCTCATCTC TCCGGTCTGA TAATACCTTC GCGGGTAATC

1101 AGACCTGCAC CAGACTCTGA GGTTGGGTTG GAGTTGTCAC CTGCCTATCC
     TCTGGACGTG GTCTGAGACT CCAACCCAAC CTCAACAGTG GACGGATAGG

1151 AGCGCCCATT AGGGTTCTCC TCTTTTCCTC CTCCAGCGTT CTCTCCCCGA
     TCGCGGGTAA TCCCAAGAGG AGAAAAGGAG GAGGTCGCAA GAGAGGGGCT

1201 CTAGACCCAC CCAACCACCT CGCCAATCCC TGTGCCCTCG CTGACTCACC
     GATCTGGGTG GGTTGGTGGA GCGGTTAGGG ACACGGGAGC GACTGAGTGG

1251 TTCTCATTTT CTCTCAGACC ACTGGGGGCT GTCATCCTCA CCTGTAGAAA
     AAGAGTAAAA GAGAGTCTGG TGACCCCCGA CAGTAGGAGT GGACATCTTT

1301 GGTGGATGCT CAGGGAGAGG AAGTCTGTCA CAGATGAGAG CTCCTCCTCG
     CCACCTACGA GTCCCTCTCC TTCAGACAGT GTCTACTCTC GAGGAGGAGC

1351 TGGATGGTGG CTATCAGAGC CCGAGAGACA CCAGGCATCC TCGCCCAGAG
     ACCTACCACC GATAGTCTCG GGCTCTCTGT GGTCCGTAGG AGCGGGTCTC

1401 GATTTGCTCC GCCCTGAAAG GGGTGTGGTG TCAAGCGGCG CAGGGTTCTC
     CTAAACGAGG CGGGACTTTC CCCACACCAC AGTTCGCCGC GTCCCAAGAG

1451 TTCCAAGGTG GGTGCCAACC AACCCAACGC GTATTGGACC TCAAGCATTC
     AAGGTTCCAC CCACGGTTGG TTGGGTTGCG CATAACCTGG AGTTCGTAAG

1501 CAGGGATGTG GCTCCCTCTG TTTGCAGAAA GTCCAATGAA CCAGCTTTGA
     GTCCCTACAC CGAGGGAGAC AAACGTCTTT CAGGTTACTT GGTCGAAACT

1551 TAGCATGCAT CCTTCGATAG CTCAGCTGGT AGAGCGGAGG ACTGTAGATT
     ATCGTACGTA GGAAGCTATC GAGTCGACCA TCTCGCCTCC TGACATCTAA

1601 GTATAGACAT TTGCGGACAT CCTTAGGTCG CTGGTTCGAT TCCAGCTCGA
     CATATCTGTA AACGCCTGTA GGAATCCAGC GACCAAGCTA AGGTCGAGCT

1651 AGGAAGTGCG TGATGCTTTT GGTTAAAAGC CCTGCAGCTT CCAAGTAGTA
     TCCTTCACGC ACTACGAAAA CCAATTTTCG GGACGTCGAA GGTTCATCAT
```

```
                10         20         30         40         50
                 |          |          |          |          |
1701 ACCACACTCT CCCGGGCAAA ACACCCACGA AGTCTTTCCT GATCACCTAG
     TGGTGTGAGA GGGCCCGTTT TGTGGGTGCT TCAGAAAGGA CTAGTGGATC

1751 CTTCCCACGC CTTGCTTCTA CTTTGTGACC CCACTAATCC CTCTATTCAT
     GAAGGGTGCG GAACGAAGAT GAAACACTGG GGTGATTAGG GAGATAAGTA

1801 GCTGACCACT ACTTCCCCAC CCTGTGCTCC TTCGCTTTTC TTCACAGACT
     CGACTGGTGA TGAAGGGGTG GGACACGAGG AAGCGAAAAG AAGTGTCTGA

1851 GCTCTCGAGC TTGAGCATCT CCACTGGCTG TGACAAAACC GCTGTGCTCA
     CGAGAGCTCG AACTCGTAGA GGTGACCGAC ACTGTTTTGG CGACACGAGT

1901 CCTTTGACAG AGCTCTCCTG ACCAGGTGGG CAAAGCCTGG AAGGTCAAGT
     GGAAACTGTC TCGAGAGGAC TGGTCCACCC GTTTCGGACC TTCCAGTTCA

1951 TCCCTGGAGA ACGTCAAGCT T
     AGGGACCTCT TGCAGTTCGA A
```

Figure 23. Nucleotide sequence of the $tRNA^{Tyr}$ gene within pM6IT-E.

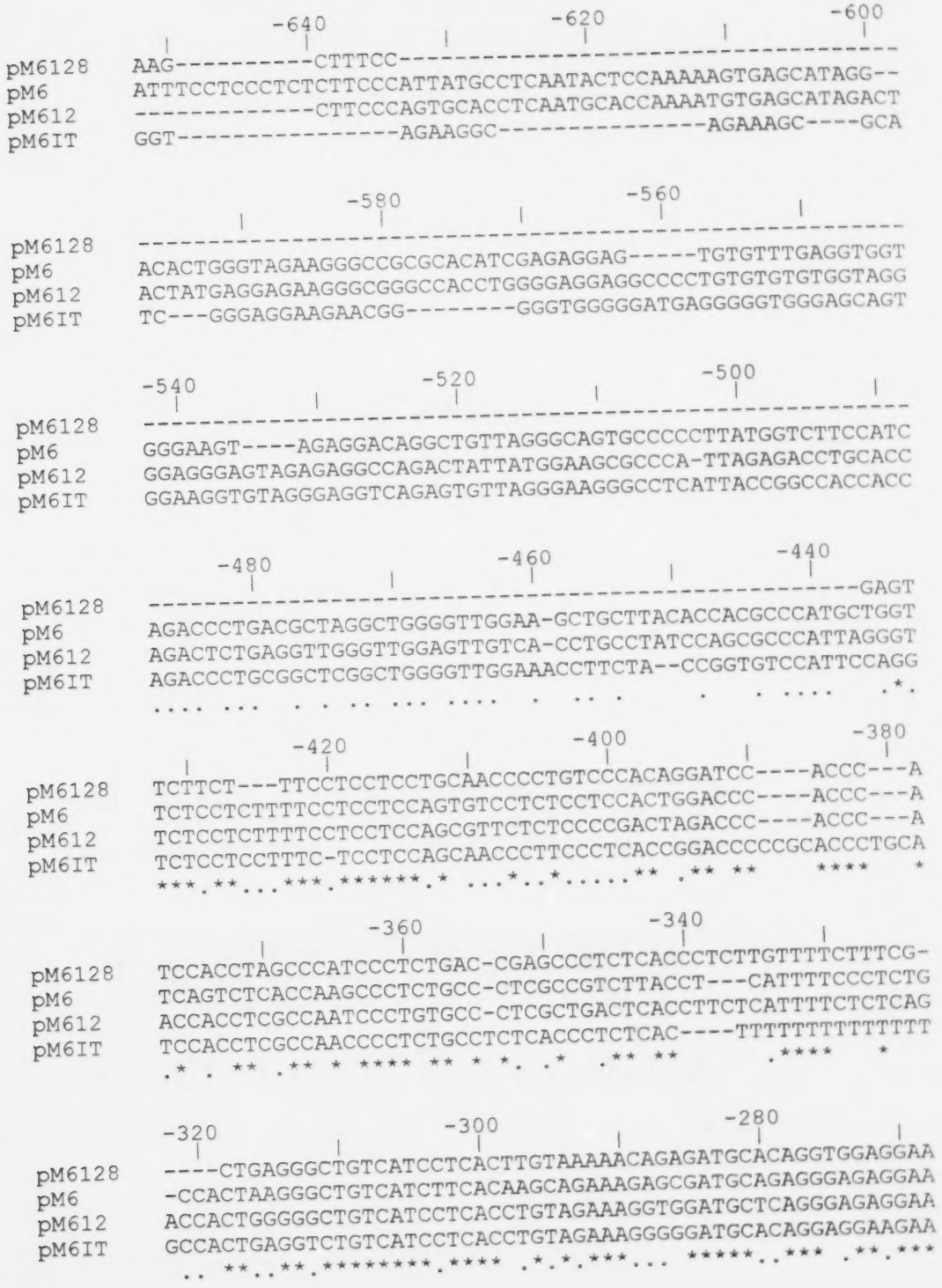
The nucleotide sequence of the 1437 bp *Eco*RI DNA fragment from λHtM6 is shown. The upper strand of sequence depicts the non-coding strand of DNA. The 6-4 $tRNA^{Tyr}$ gene which starts at position 1014 is shown in bold, while the intervening sequence is underlined. A transcription termination signal of 4 T residues is found at position 1120. The nucleotide sequence initially determined by MacPherson (1988) is indicated with dashed lines.

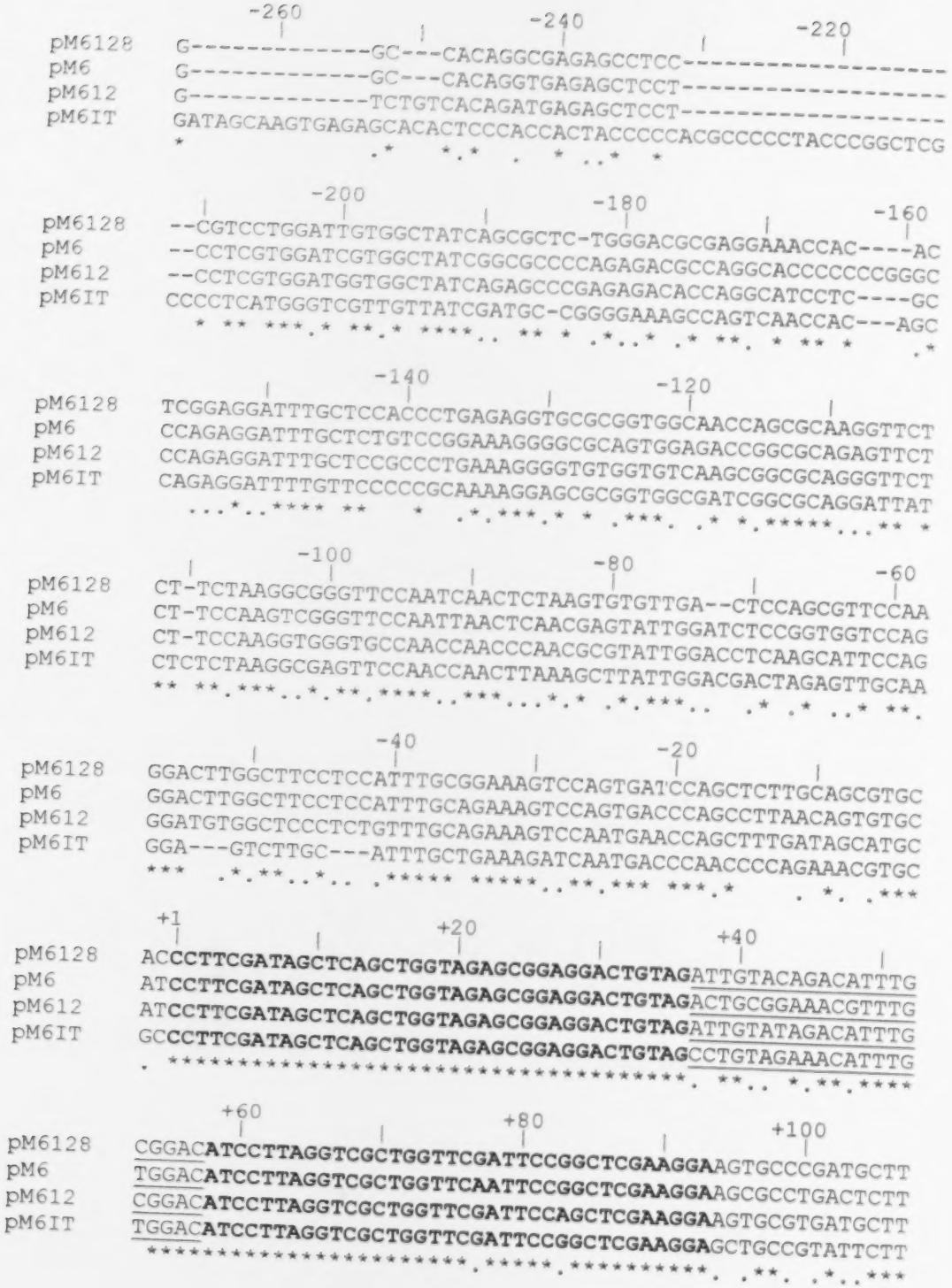
```
                10         20         30         40         50
                |          |          |          |          |
  1 GAATTCAGGG GCATAATCAT AGCTCACTGT AGCCTTGAAC TCCTGGCCTC
    CTTAAGTCCC CGTATTAGTA TCGAGTGACA TCGGAACTTG AGGACCGGAG

 51 AAGCAATCCT CCCACCTCAG CCTCCCAAGT AGCTGGGACT ACAAGCTTGT
    TTCGTTAGGA GGGTGGAGTC GGAGGGTTCA TCGACCCTGA TGTTCGAACA

101 GCTACCACAC CCAGCTAATT TTTGTAATTT TTGTAAAGAT AAGGTTTTGC
    CGATGGTGTG GGTCGATTAA AAACATTAAA AACATTTCTA TTCCAAAACG

151 CGTGTTGCTC AGGCTGGTCC TTGCCCCTTT GCTTTTTCAG AGCCCACAGA
    GCACAACGAG TCCGACCAGG AACGGGGAAA CGAAAAAGTC TCGGGTGTCT

201 GCCTTGTCCT ACTTCTAGAA GTTGCAAGTG GCAAAATATT TCTGATTGGT
    CGGAACAGGA TGAAGATCTT CAACGTTCAC CGTTTTATAA AGACTAACCA

251 TTGCAGAGGA GGGAAGCCAA CCACCAGATT GGCCCCGAGG GAGGAGGGGT
    AACGTCTCCT CCCTTCGGTT GGTGGTCTAA CCGGGGCTCC CTCCTCCCCA

301 ATTACATGTG CCTCTGCAAT CCCTGGGAGG GGTCTCCTCA CCTAATTCTA
    TAATGTACAC GGAGACGTTA GGGACCCTCC CCAGAGGAGT GGATTAAGAT

351 TTTGTTCTTT CCCTCACTTC CCAGTACACC TCAACGGGCC AATACGTGAA
    AAACAAGAAA GGGAGTGAAG GGTCATGTGG AGTTGCCCTG TTATGCACTT

401 CTTAGGATAC TGTAGGTAGA AGGCAGAAAG CGCATCGGGA GGAAGAACGG
    GAATCGTATG ACATCCATCT TCCGTCTTTC GCGTAGCCCT CCTTCTTGCC

451 GGGTGGGGGA TGAGGGGGTG GGAGCAGTGG AAGGTGTAGG GAGGTCAGAG
    CCCACCCCCT ACTCCCCCAC CCTCGTCACC TTCCACATCC CTCCAGTCTC

501 TGTTAGGGAA GGGCCTCATT ACCGGCCACC ACCAGACCCT GCGGCTCGGC
    ACAATCCCTT CCCGGAGTAA TGGCCGGTGG TGGTCTGGGA CGCCGAGCCG

551 TGGGGTTGGA AACCTTCTAC CGGTGTCCAT TCCAGGTCTC CTCCTTTCTC
    ACCCCAACCT TTGGAAGATG GCCACAGGTA AGGTCCAGAG GAGGAAAGAG

601 CTCCAGCAAC CCTTCCCTCA CCGGACCCCC GCACCCTGCA TCCACCTCGC
    GAGGTCGTTG GGAAGGGAGT GGCCTGGGGG CGTGGGACGT AGGTGGAGCG

651 CAACCCCTCT GCCTCTCACC CTCTCACTTT TTTTTTTTTT TGCCACTGAG
    GTTGGGGAGA CGGAGAGTGG GAGAGTGAAA AAAAAAAAAA ACGGTGACTC

701 GTCTGTCATC CTCACCTGTA GAAAGGGGGA TGCACAGGAG GAAGAAGATA
    CAGACAGTAG GAGTGGACAT CTTTCCCCCT ACGTGTCCTC CTTCTTCTAT

751 GCAAGTGAGA GCACACTCCC ACCACTACCC CCACGCCCCC TACCCGGCTC
    CGTTCACTCT CGTGTGAGGG TGGTGATGGG GGTGCGGGGG ATGGGCCGAG

801 GCCCCTCATG GGTCGTTGTT ATCGATGCCG GGGAAAGCCA GTCAACCACA
    CGGGGAGTAC CCAGCAACAA TAGCTACGGC CCCTTTCGGT CAGTTGGTGT

851 GCCAGAGGAT TTTGTTCCCC CGCAAAAGGA GCGCGGTGGC GATCGGCGCA
    CGGTCTCCTA AAACAAGGGG GCGTTTTCCT CGCGCCACCG CTAGCCGCGT

901 GGATTATCTC TCTAAGGCGA GTTCCAACCA ACTTAAAGCT TATTGGACGA
    CCTAATAGAG AGATTCCGCT CAAGGTTGGT TGAATTTCGA ATAACCTGCT
```

```
                10          20          30          40          50
                 |           |           |           |           |
 951 CTAGAGTTGC AAGGAGTCTT GCATTTGCTG AAAGATCAAT GACCCAACCC
     GATCTCAACG TTCCTCAGAA CGTAAACGAC TTTCTAGTTA CTGGGTTGGG

1001 CAGAAACGTG CGCCCTTCGA TAGCTCAGCT GGTAGAGCGG AGGACTGTAG
     GTCTTTGCAC GCGGGAAGCT ATCGAGTCGA CCATCTCGCC TCCTGACATC

1051 CCTGTAGAAA CATTTGTGGA CATCCTTAGG TCGCTGGTTC GATTCCGGCT
     GGACATCTTT GTAAACACCT GTAGGAATCC AGCGACCAAG CTAAGGCCGA

1101 CGAAGGAGCT GCCGTATTCT TTTGCACACG CACGCACCAA AACTACGTGG
     GCTTCCTCGA CGGCATAAGA AAACGTGTGC GTGCGTGGTT TTGATGCACC

1151 CTGCATCTCT GCCTGGTCAA AGGCTTTGCC AGCCAGCATC CACACTCTCC
     GACGTAGAGA CGGACCAGTT TCCGAAACGG TCGGTCGTAG GTGTGAGAGG

1201 CAGGAGAAAC CTAGCAAGGC CTTTCCGGAT TACCCAGCTT CCCACAGCCT
     GTCCTCTTTG GATCGTTCCG GAAAGGCCTA ATGGGTCGAA GGGTGTCGGA

1251 ATGCTGTGGC CTTGGGAGTC TGCTCATTCT TCAAGTCATT GCTTTCTCTA
     TACGACACCG GAACCCTCAG ACGAGTAAGA AGTTCAGTAA CGAAAGAGAT

1301 CTATCTTCAA ATTTTTTATA TAATCTGGGA TTTTTATCTC CAAGTCATTG
     GATAGAAGTT TAAAAAATAT ATTAGACCCT AAAAATAGAG GTTCAGTAAC

1351 ATTTTTATGG CCATTAGGTG ACACTATAAC TGATATTCTT ATGAAACTAG
     TAAAAATACC GGTAATCCAC TGTGATATTG ACTATAAGAA TACTTTGATC

1401 ATGAAATATT TGAATAACAT GTTAGAAAAT TGAATTC
     TACTTTATAA ACTTATTGTA CAATCTTTTA ACTTAAG
```

Figure 24. Nucleotide sequence alignment of the λHtM6 tRNA^{Tyr} genes and their flanking sequences.

The nucleotide sequence alignment depicts the non-coding strands of DNA from each of the four recombinant plasmids. One kb of sequence from pM6, pM612 and pM6IT was used for the alignment, however, only 855 bp of sequence from pM6128 was available for the alignment. The pM6128, pM6, pM612, and pM6IT plasmids carry the 6-1, 6-2, 6-3, and 6-4 tRNA^{Tyr} genes, respectively. The tDNA sequences are shown in bold type and their intervening sequences are underlined. The positions that are perfectly conserved are indicated by an asterisk (*), while the positions that are well conserved are indicated with a period (.).


-640
-620
-600
pM6128 AAG----------CTTTCC----------
pM6 ATTTCCTCCCTCTCTTCCCATTATGCCTCAATACTCCAAAAAGTGAGCATAGG--
pM612 ----------CTTCCCAGTGCACCTCAATGCACCAAAATGTGAGCATAGACT
pM6IT GGT----------AGAAGGC----------AGAAAGC----GCA
-580
-560
pM6128 ----------
pM6 ACACTGGGTAGAAGGGCCGCGCACATCGAGAGGAG-----TGTGTTTGAGGTGGT
pM612 ACTATGAGGAGAAGGGCGGGCCACCTGGGGAGGAGGCCCCTGTGTGTGTGGTAGG
pM6IT TC---GGGAGGAAGAACGG--------GGGTGGGGGATGAGGGGGTGGGAGCAGT
-540
-520
-500
pM6128 ----------
pM6 GGGAAGT----AGAGGACAGGCTGTTAGGGCAGTGCCCCCTTATGGTCTTCCATC
pM612 GGAGGGAGTAGAGAGGCCAGACTATTATGGAAGCGCCCA-TTAGAGACCTGCACC
pM6IT GGAAGGTGTAGGGAGGTCAGAGTGTTAGGGAAGGGCCTCATTACCGGCCACCACC
-480
-460
-440
pM6128 ----------GAGT
pM6 AGACCCTGACGCTAGGCTGGGGTTGGAA-GCTGCTTACACCACGCCCATGCTGGT
pM612 AGACTCTGAGGTTGGGTTGGAGTTGTCA-CCTGCCTATCCAGCGCCCATTAGGGT
pM6IT AGACCCTGCGGCTCGGCTGGGGTTGGAAACCTTCTA--CCGGTGTCCATTCCAGG
....*.
-420
-400
-380
pM6128 TCTTCT---TTCCTCCTCCTGCAACCCCTGTCCCACAGGATCC----ACCC---A
pM6 TCTCCTCTTTTCCTCCTCCAGTGTCCTCTCCTCCACTGGACCC----ACCC---A
pM612 TCTCCTCTTTTCCTCCTCCAGCGTTCTCTCCCCGACTAGACCC----ACCC---A
pM6IT TCTCCTCCTTTC-TCCTCCAGCAACCCTTCCCTCACCGGACCCCCGCACCCTGCA
.**....******.* ...*..*.....** .** ** **** *
-360
-340
pM6128 TCCACCTAGCCCATCCCTCTGAC-CGAGCCCTCTCACCCTCTTGTTTTCTTTCG-
pM6 TCAGTCTCACCAAGCCCTCTGCC-CTCGCCGTCTTACCT---CATTTTCCCTCTG
pM612 ACCACCTCGCCAATCCCTGTGCC-CTCGCTGACTCACCTTCTCATTTTCTCTCAG
pM6IT TCCACCTCGCCAACCCCTCTGCCTCTCACCCTCTCAC----TTTTTTTTTTTTT
.* . ** .** * **** ** * *. .* .** ** .**** *
-320
-300
-280
pM6128 ----CTGAGGGCTGTCATCCTCACTTGTAAAAACAGAGATGCACAGGTGGAGGAA
pM6 -CCACTAAGGGCTGTCATCTTCACAAGCAGAAAGAGCGATGCAGAGGGAGAGGAA
pM612 ACCACTGGGGGCTGTCATCCTCACCTGTAGAAAGGTGGATGCTCAGGGAGAGGAA
pM6IT GCCACTGAGGTCTGTCATCCTCACCTGTAGAAAGGGGGATGCACAGGAGGAAGAA
.. **..**.********.**** .*.*.***... *****..*** .**.***


-260
-240
-220
pM6128 G----------GC---CACAGGCGAGAGCCTCC----------
pM6 G----------GC---CACAGGTGAGAGCTCCT----------
pM612 G----------TCTGTCACAGATGAGAGCTCCT----------
pM6IT GATAGCAAGTGAGAGCACACTCCCACCACTACCCCCACGCCCCCTACCCGGCTCG
-200
-180
-160
pM6128 --CGTCCTGGATTGTGGCTATCAGCGCTC-TGGGACGCGAGGAAACCAC----AC
pM6 --CCTCGTGGATCGTGGCTATCGGCGCCCCAGAGACGCCAGGCACCCCCCCGGGC
pM612 --CCTCGTGGATGGTGGCTATCAGAGCCCGAGAGACACCAGGCATCCTC----GC
pM6IT CCCCTCATGGGTCGTTGTTATCGATGC-CGGGGAAAGCCAGTCAACCAC---AGC
-140
-120
pM6128 TCGGAGGATTTGCTCCACCCTGAGAGGTGCGCGGTGGCAACCAGCGCAAGGTTCT
pM6 CCAGAGGATTTGCTCTGTCCGGAAAGGGGCGCAGTGGAGACCGGCGCAGAGTTCT
pM612 CCAGAGGATTTGCTCCGCCCTGAAAGGGGTGTGGTGTCAAGCGGCGCAGGGTTCT
pM6IT CAGAGGATTTTGTTCCCCCGCAAAAGGAGCGCGGTGGCGATCGGCGCAGGATTAT
-100
-80
-60
pM6128 CT-TCTAAGGCGGGTTCCAATCAACTCTAAGTGTGTTGA--CTCCAGCGTTCCAA
pM6 CT-TCCAAGTCGGGTTCCAATTAACTCAACGAGTATTGGATCTCCGGTGGTCCAG
pM612 CT-TCCAAGGTGGGTGCCAACCAACCCAACGCGTATTGGACCTCAAGCATTCCAG
pM6IT CTCTCTAAGGCGAGTTCCAACCAACTTAAAGCTTATTGGACGACTAGAGTTGCAA
-40
-20
pM6128 GGACTTGGCTTCCTCCATTTGCGGAAAGTCCAGTGATCCAGCTCTTGCAGCGTGC
pM6 GGACTTGGCTTCCTCCATTTGCAGAAAGTCCAGTGACCCAGCCTTAACAGTGTGC
pM612 GGATGTGGCTCCCTCTGTTTGCAGAAAGTCCAATGAACCAGCTTTGATAGCATGC
pM6IT GGA---GTCTTGC---ATTTGCTGAAAGATCAATGACCCAACCCCAGAAACGTGC
+1
+20
+40
pM6128 ACCCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAGATTGTACAGACATTTG
pM6 ATCCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAGACTGCGGAAACGTTTG
pM612 ATCCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAGATTGTATAGACATTTG
pM6IT GCCCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAGCCTGTAGAAACATTTG
+60
+80
+100
pM6128 CGGACATCCTTAGGTCGCTGGTTCGATTCCGGCTCGAAGGAAGTGCCCGATGCTT
pM6 TGGACATCCTTAGGTCGCTGGTTCAATTCCGGCTCGAAGGAAGCGCCTGACTCTT
pM612 CGGACATCCTTAGGTCGCTGGTTCGATTCCAGCTCGAAGGAAGTGCGTGATGCTT
pM6IT TGGACATCCTTAGGTCGCTGGTTCGATTCCGGCTCGAAGGAGCTGCCGTATTCTT

```
                    +120                +140                +160
                     |                   |                   |
pM6128   TTGCATGCAATGCCACCTGGTGC--CTGGTCAAACGCCCTGCAGCCTCCAACTAG
pM6      TTGCGCACAATGCTGCCTGG-----CTG--CA-----CCTG-TTCCTC-GTC-AA
pM612    TTG-GTTAAAAGC--CCTG------CAG--C-----------TTCCAA-GT--AG
pM6IT    TTGCACACGCACGCACCAAAA-CTACGTGGCTG-CATC--TCTGCCTG-GTCAAA
         *** . . . .   **..     *..  *          ..**.  ..  *.

                    +180                +200
                     |                   |                   |
pM6128   TATCCACCCACACCCTCCCAGTCAAAAC--CCAGAGAAACCTTTCC--TGATCAC
pM6      AGACCTT--GCAGCCTTCCAGTCATAACTAC-------ACTTTCCCCAGGAAAAC
pM612    TAACCAC--ACT--CTCCCGGGCAAAACACCCACGAAGTCTTTCC---TGATCAC
pM6IT    GG------CTTTGCCAGCCAG------CATCCACA----CTCTCCCAGGAGAAAC
         ..       . .  *. **.*      *  *         *  * *   .... **

         +220                +240                +260
          |                   |                   |                   |
pM6128   CTGG---TTTCCACACCTGTGCTGTGGCCAGGAAACACGCCCGTAAGCCCACTC-
pM6      CCAGCAAAATCCT-GCCT-TTCCTGATCACAGG------CCTGGGAGCCTATTC-
pM612    CTAGCTTC--CCACGCCT-TGCTTCTACTTTGTGAC-C-CCACTAATCC--CTCT
pM6IT    CTAGCAAGG-CCTTTCCGGA----TTACCCAGCTTCCCAC-----AGCCTATGCT
         *..*.     **. .**. . .   ..*  .*   . . *.    *.**   .*

              +280                +300                +320
               |                   |                   |                   |
pM6128   ATTCTTCCCACAGTCCAGGGACAGGTACTC-TTCAACG---CAAA---GGGCCC-
pM6      ATTCTTCCCG-AATCCT----CATAG--TC-TTCAGCT---CAAAACAGTGCCC-
pM612    ATTCATGCTG-A--CCA----CTA-----C-TTCCCCA---CC---CTGTGCTC-
pM6IT    GT----------GGCCTTGGGAGTCTGCTCATTCTTCAAGTCATTGCT-TTCTCT
         .*.. . .     **.     ..     .* ***  *.   *  .    ..* *

                    +340                +360                +380
                     |                   |                   |                   |
pM6128   -CTTGCTTCTGCTTTGTGACCCAGTCAGCCCCTCTCCCCAACCCCCTTCGCACCC
pM6      -CTT------GCCTTGTGACCCAGCCAGACCC------CAAT-TCGT--GC--CC
pM6      -CTT-C----GCTTTTCTTCACAGACTGCTCT------CGAGCTTGA--GCATCT
pM6IT    ACTATCTTCAAATTTTTTATATAATCTG-----------GGATTTTTATCTCCA

                    +400                +420
                     |                   |                   |
pM6128   CCACCATCCAGAGCTCTTTCACTTTTATC--CACAGACTG--CTCTG-GGTGCTG
pM6      CCACCATCCTACGCCTTTCCACTTGTATTTACAA--ACTG--CCCACCCATCC--
pM612    CCACTGGCTGTGACAAAACCGCTGTGCTCACCTTTGACAGAGCTCTCCTGACCAG
pM6IT    AGTCATTGATTTTTATGGCCATTAGGTGACACTATAACTGA-------TATTCTT

         +440                +460                +480
          |                   |                   |                   |
pM6128   AGGGCCATGGACTCTAGCAACATTGTGTGCTCACCATT-GATAGATC-----T
pM6      -----C----CCTG-------------TC---CCAGA-C--CCGTCAAGCTT
pM612    GTGGGCAAAGCCTGGAAGGTCAA---GTTC---CCTGG-AGAACGTCAAGCTT
pM6IT    ATG-----AAACTAGATGAAATATTTGAA-TAACATGTTAGAAAATTGAATTC
```

Figure 25. Nucleotide sequences of the $tRNA^{Tyr}$ genes cloned into pBS.

The sequences depict the non-coding strand of DNA. For the purpose of identification the exon sequences have been separated from the intron sequences. The tDNA sequence designated (1) for each $tRNA^{Tyr}$ gene represent the wild-type sequences. The mutations in the pBS clones are indicated by underlined positions. The 4-1 $tRNA^{Tyr}$ gene sequences (2) and (3) are from pBS clones pJM4 #60 and #527, respectively. The 6-1 $tRNA^{Tyr}$ gene sequence (2) is from pBS clone pM6128 #272. The 6-3 $tRNA^{Tyr}$ gene sequence (2) is from pBS clones pM612 #50. The M6IT $tRNA^{Tyr}$ gene sequences (2) and (3) are from pBS clones pM6IT #39 and #55, respectively. The 6-2 $tRNA^{Tyr}$ gene sequence (2) is from pBS clone pM6 #436, which contains two $tRNA^{Tyr}$ genes in tandem.

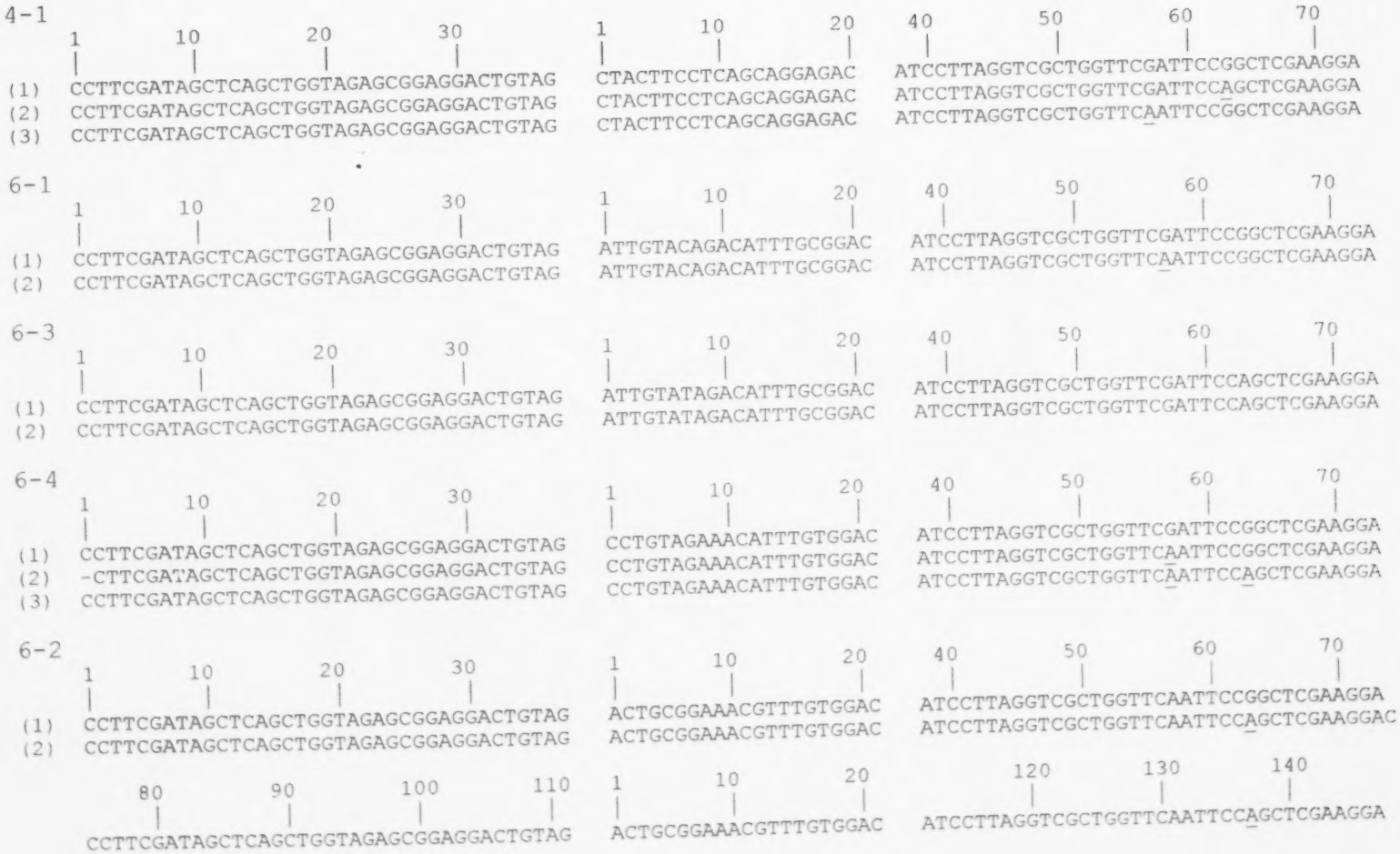

4-1
1 10 20 30
(1) CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAG
(2) CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAG
(3) CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAG
1 10 20
CTACTTCCTCAGCAGGAGAC
CTACTTCCTCAGCAGGAGAC
CTACTTCCTCAGCAGGAGAC
40 50 60 70
ATCCTTAGGTCGCTGGTTCGATTCCGGCTCGAAGGA
ATCCTTAGGTCGCTGGTTCGATTCCAGCTCGAAGGA
ATCCTTAGGTCGCTGGTTCAATTCCGGCTCGAAGGA
6-1
1 10 20 30
(1) CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAG
(2) CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAG
1 10 20
ATTGTACAGACATTTGCGGAC
ATTGTACAGACATTTGCGGAC
40 50 60 70
ATCCTTAGGTCGCTGGTTCGATTCCGGCTCGAAGGA
ATCCTTAGGTCGCTGGTTCAATTCCGGCTCGAAGGA
6-3
1 10 20 30
(1) CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAG
(2) CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAG
1 10 20
ATTGTATAGACATTTGCGGAC
ATTGTATAGACATTTGCGGAC
40 50 60 70
ATCCTTAGGTCGCTGGTTCGATTCCAGCTCGAAGGA
ATCCTTAGGTCGCTGGTTCGATTCCAGCTCGAAGGA
6-4
1 10 20 30
(1) CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAG
(2) -CTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAG
(3) CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAG
1 10 20
CCTGTAGAAACATTTGTGGAC
CCTGTAGAAACATTTGTGGAC
CCTGTAGAAACATTTGTGGAC
40 50 60 70
ATCCTTAGGTCGCTGGTTCGATTCCGGCTCGAAGGA
ATCCTTAGGTCGCTGGTTCAATTCCGGCTCGAAGGA
ATCCTTAGGTCGCTGGTTCAATTCCAGCTCGAAGGA
6-2
1 10 20 30
(1) CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAG
(2) CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAG
80 90 100 110
CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGTAG
1 10 20
ACTGCGGAAACGTTTGTGGAC
ACTGCGGAAACGTTTGTGGAC
1 10 20
ACTGCGGAAACGTTTGTGGAC
40 50 60 70
ATCCTTAGGTCGCTGGTTCAATTCCGGCTCGAAGGA
ATCCTTAGGTCGCTGGTTCAATTCCAGCTCGAAGGAC
120 130 140
ATCCTTAGGTCGCTGGTTCAATTCCAGCTCGAAGGA

Figure 26. Restriction endonuclease maps of the recombinant plasmids containing the tRNA genes from λHtM4.

These restriction maps were generated from the nucleotide sequence of pJM4, a recombinant plasmid which contains a 2.4 kb *Hin*dIII/*Eco*RI DNA fragment. The tRNA genes are each represented by an arrow enclosed in a box, with the point of the arrow indicating the direction of gene transcription The recombinant plasmids pJM4-HA and pJM4-MA both have 0.26 kb of 3' flanking sequence, but pJM4-HA has more 5' flanking sequence. The recombinant plasmids pJM4 #60 and pJM4 #527, which differ by a single mutation, both have no human flanking sequences on either side of the $tRNA^{Tyr}$ gene. The recombinant plasmids pJMS42-AE and pJMS42-AS both have 250 bp of 5' flanking sequence, but pJMS42-AS has less 3' flanking sequence. The recombinant plasmid pDSALA has 31 bp of 5' flanking sequence upstream of the $tRNA^{Ala}$ gene.

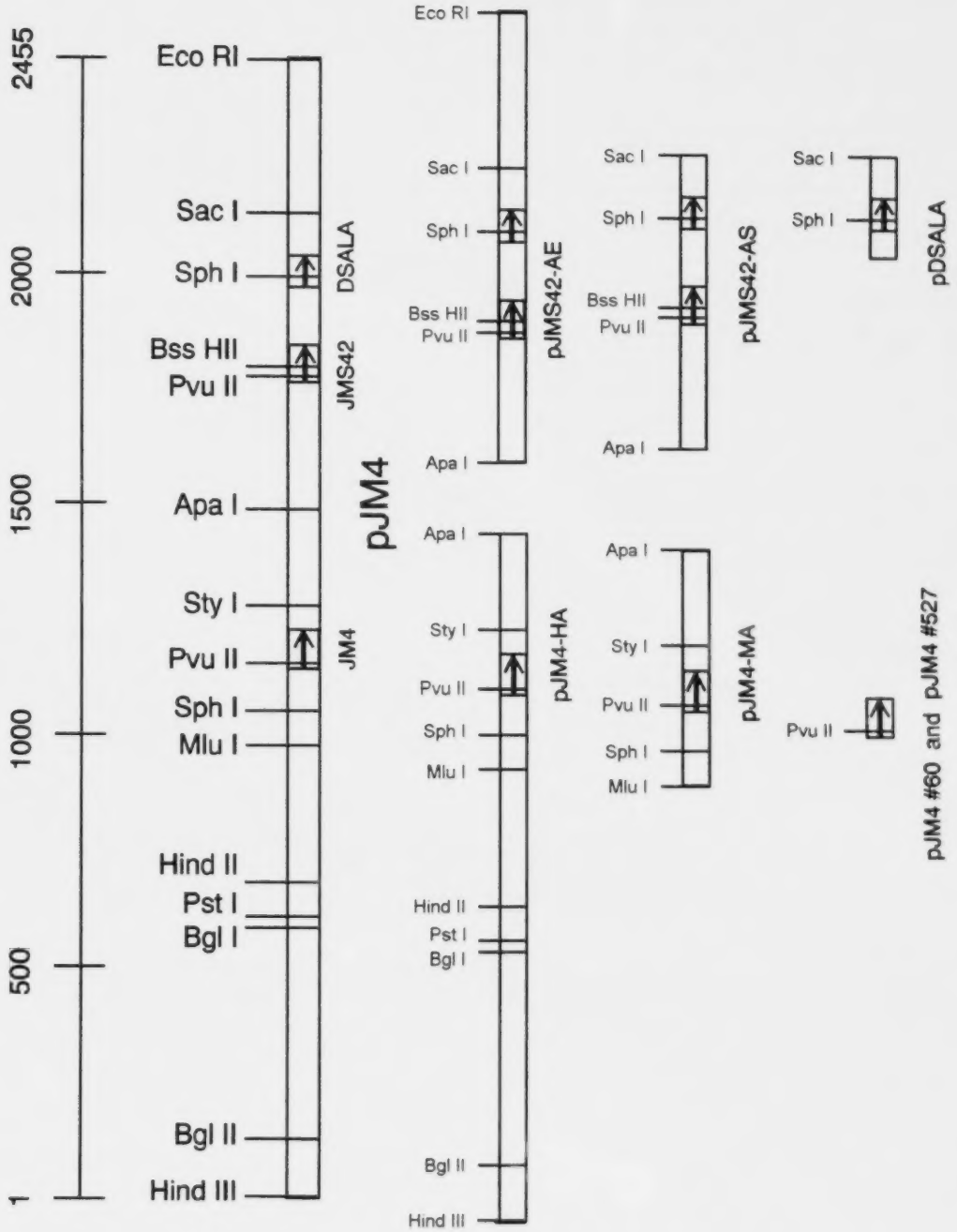

2455
2000
1500
1000
500
1
Eco RI
Sac I
Sph I
Bss HII
Pvu II
Apa I
Sty I
Pvu II
Sph I
Mlu I
Hind II
Pst I
Bgl I
Bgl II
Hind III
DSALA
JMS42
JM4
pJM4
Eco RI
Sac I
Sph I
Bss HII
Pvu II
Apa I
pJMS42-AE
Sac I
Sph I
Bss HII
Pvu II
Apa I
pJMS42-AS
Sac I
Sph I
pDSALA
Apa I
Sty I
Pvu II
Sph I
Mlu I
Hind II
Pst I
Bgl I
Bgl II
Hind III
pJM4-HA
Apa I
Sty I
Pvu II
Sph I
Mlu I
pJM4-MA
Pvu II
pJM4 #60 and pJM4 #527

Figure 27. Restriction endonuclease maps of recombinant plasmids containing the 6-1 $tRNA^{Tyr}$ gene.

These restriction maps were generated from the nucleotide sequence of the pM6128-HB, a recombinant plasmid which contains an 855 bp *Hin*dIII/*Bgl*II DNA fragment from λHtM2. The $tRNA^{Tyr}$ gene is represented by an arrow enclosed in a box, with the point of the arrow indicating the direction of gene transcription. The recombinant plasmid pM6128-HB has 395 bp of 5' flanking sequence, but 0.65 kb less 3' flanking sequence than pM6128. The recombinant plasmid pM6128-S has 61 bp of 5' flanking sequence. The recombinant plasmid pM6128 #272 has no human flanking sequences on either side of the tRNA gene.

1
100
200
300
400
500
600
700
800
855
Hind III
Bam HI
Hind II
Sty I
Apa LI
Pvu II
Sph I
Pst I
Apa I
Sac I
Sty I
Bgl II
pM6128-HB
Sty I
Sac I
Apa I
Pst I
Sph I
Apa LI
Pvu II
Sty I
pM6128-S
Pvu II
pM6128 #272

Figure 28. Restriction endonuclease maps of recombinant plasmids containing the 6-2 $tRNA^{Tyr}$ gene.

These restriction maps were generated from the nucleotide sequence of the pM6, the recombinant plasmid which contains the 1390 bp *Hin*dIII fragment of λHtM6 subcloned into plasmid vector pUC118. The tRNA gene is shown as an arrow enclosed in a box, with the point of the arrow indicating the direction of gene transcription. The recombinant plasmid pM6 has 997 bp of 5' flanking sequence. The recombinant plasmid pM6 #436, which has no human flanking sequences, contains two tandem *in vitro* mutated 6-2 $tRNA^{Tyr}$ genes as a result of cloning tDNA sequences that had been amplified by PCR.

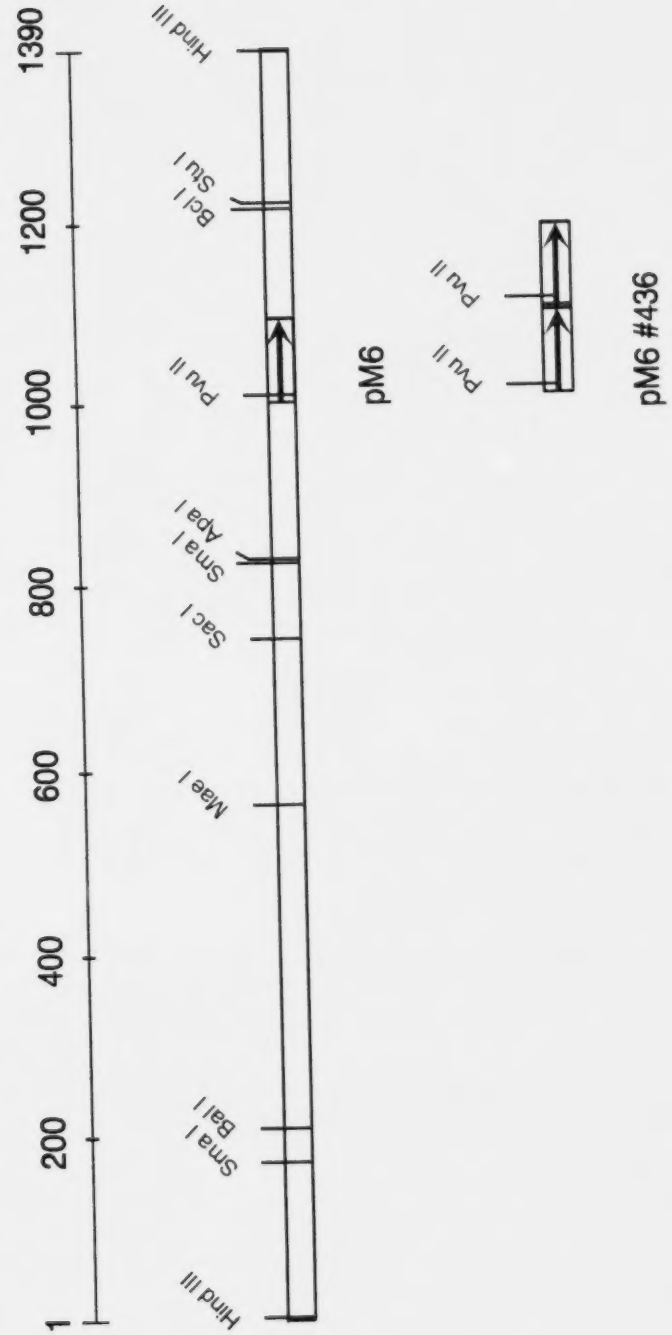

1
200
400
600
800
1000
1200
1390
Hind III
Sma I
Bal I
Mae I
Sac I
Sma I
Apa I
Pvu II
Bcl I
Stu I
Hind III
pM6
Pvu II
Pvu II
pM6 #436

Figure 29. Restriction endonuclease maps of the recombinant plasmids containing the 6-3 $tRNA^{Tyr}$ gene.

These restriction maps were generated from the nucleotide sequence of pM612, a recombinant plasmid which contains a 1971 bp *Hin*dIII DNA fragment from λHtM6. The tRNA genes are each represented by an arrow enclosed in a box, with the point of the arrow indicating the direction of gene transcription The recombinant plasmids pM612, pM612-S and pM612-N have 1561, 219 and 2 bp of 5' flanking sequence, respectively. The recombinant plasmid pM612 #50 has no human flanking sequences on either side of the $tRNA^{Tyr}$ gene.

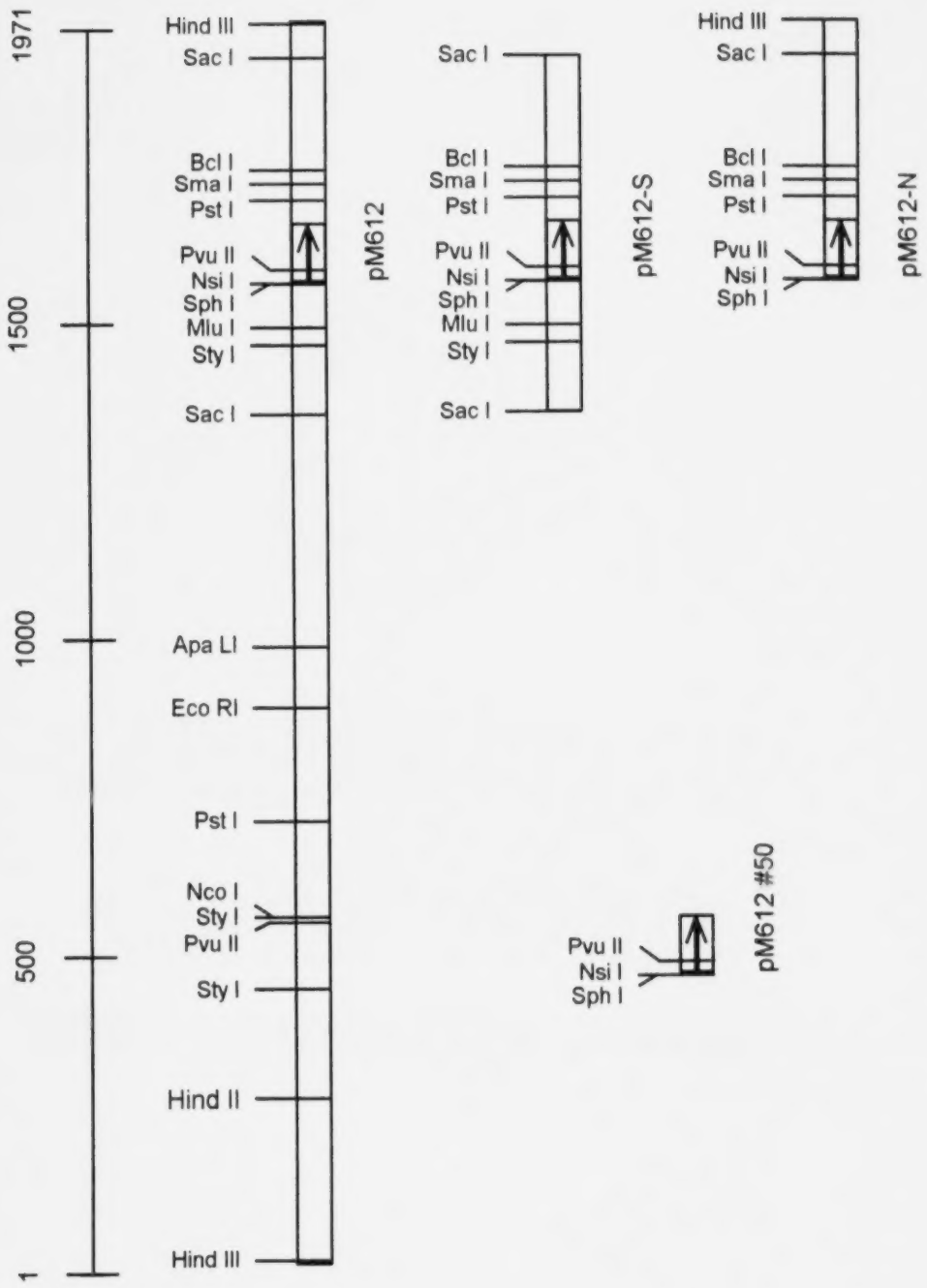
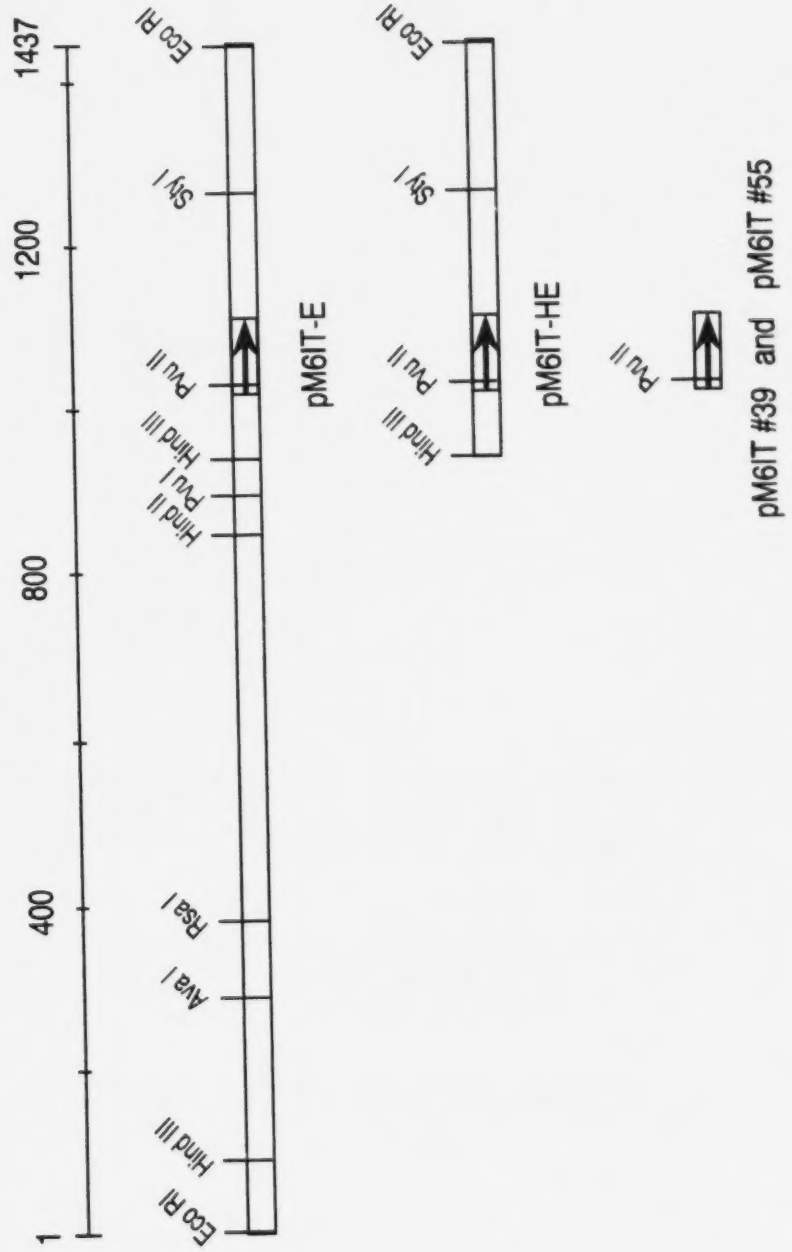

1971
1500
1000
500
1
Hind III
Sac I
Bcl I
Sma I
Pst I
Pvu II
Nsi I
Sph I
Mlu I
Sty I
Sac I
Apa LI
Eco RI
Pst I
Nco I
Sty I
Pvu II
Sty I
Hind II
Hind III
pM612
Sac I
Bcl I
Sma I
Pst I
Pvu II
Nsi I
Sph I
Mlu I
Sty I
Sac I
pM612-S
Hind III
Sac I
Bcl I
Sma I
Pst I
Pvu II
Nsi I
Sph I
pM612-N
Pvu II
Nsi I
Sph I
pM612 #50

Figure 30. Restriction endonuclease maps of the recombinant plasmids containing the 6-4 $tRNA^{Tyr}$ gene.

These restriction maps were generated from the nucleotide sequence of pM6IT-E, a recombinant plasmid which contains a 1437 bp *Eco*RI DNA fragment from λHtM6. The tRNA genes are each represented by an arrow enclosed in a box, with the point of the arrow indicating the direction of gene transcription The recombinant plasmids pM6IT-E and pM6IT-HE have 1014 and 78 bp of 5' flanking sequence, respectively. The recombinant plasmids pM6IT #39 and pM6IT #55, which differ by two mutations, have no human flanking sequences on either side of the $tRNA^{Tyr}$ gene.


1
400
800
1200
1437
Eco RI
Hind III
Ava I
Rsa I
Hind II
Pvu I
Hind III
Pvu II
Sty I
Eco RI
pM6IT-E
Eco RI
Hind III
Pvu II
Sty I
Eco RI
pM6IT-HE
Pvu II
pM6IT #39 and pM6IT #55

protocol described by Weil *et al.* (1979) consistently produced active S-100 cell extracts that had low RNase activity and very little batch-to-batch variation, especially when 293 cells were used.

All of the *in vitro* experiments described in this section were performed with the same preparation of 293 cell S-100 extract to standardize the results. The preparation of S-100 cell extract used for these *in vitro* experiments had high RNA polymerase III activity but had very low tRNA processing activity.

The plasmid vectors (pAT153, pUC118, pUC119, and pBS) used to clone gene-containing restriction fragments were tested for transcriptional activity and found not to direct the synthesis of specific RNA transcripts in mammalian cell extracts. These vectors can give rise to nonspecific RNA synthesis when a significant amount of the plasmid DNA is in the relaxed circular or linear form. This nonspecific RNA synthesis was responsible for the high molecular weight bands observed on the autoradiographs from the in vitro transcription experiments (Figures 31 - 36).

Five of the $tRNA^{Tyr}$ gene-containing plasmids, pM6128, pM6IT, pM612, pM6 and pJM4-HA, had very similar transcription efficiencies *in vitro*. The nearly identical transcription efficiencies of these gene containing plasmids is evident in lanes 3 - 7 of Figure 31, which contain similar amounts of pre-$tRNA^{Tyr}$ transcripts. The most abundant RNA species were the pre-$tRNA^{Tyr}$ transcripts (112 - 115 nt), while the minor speices were the processing intermediates. The 5' half of pre-$tRNA^{Tyr}$ was approxiamtely 43 nt and the 3' half was approxiamtely 52 nt. Similar expression levels amongst the λHtM6 $tRNA^{Tyr}$ genes were not unexpected due to the sequence homology in the 5' flanking sequences of these genes.

The transcription efficiency of the sixth $tRNA^{Tyr}$ gene, 4-2, was difficult to compare with the others due to the presence of the 4-3 $tRNA^{Ala}$ gene in the pJMS42 constructs. When the *in vitro* transcription assays were performed with templates that carried both the 4-2 and 4-3 tRNA genes, the pre-tRNAs were identical in size (112 - 115

Figure 31. RNA transcripts from *in vitro* transcription reactions directed by recombinant plasmids containing cloned human tRNA genes.

The nucleic acids extracted from these *in vitro* reactions were separated electrophoretically on 10% denaturing polyacrylamide gels and the RNA transcripts synthesized *in vitro* were visualized by 8 - 16 hours of autoradiography at -20°C. Lanes 1 and 10 contain single-stranded, radioactively labeled size markers which consisted of a mixture of 5S RNA from *E. coli* (121 nt.), $tRNA^{Phe}$ from brewer's yeast (77 nt.), and four synthetic oligonucleotides (89, 53, 41 and 22 nt.). Lane 2 is a control lane, showing the synthesis of products directed by the vector pUC118 alone. The recombinant plasmids directing the *in vitro* synthesis in lanes 3- 9 were pM6128, pM6IT, pM612, pM6, pJM4-HA, pJMS42-AE and pDSALA, respectively.

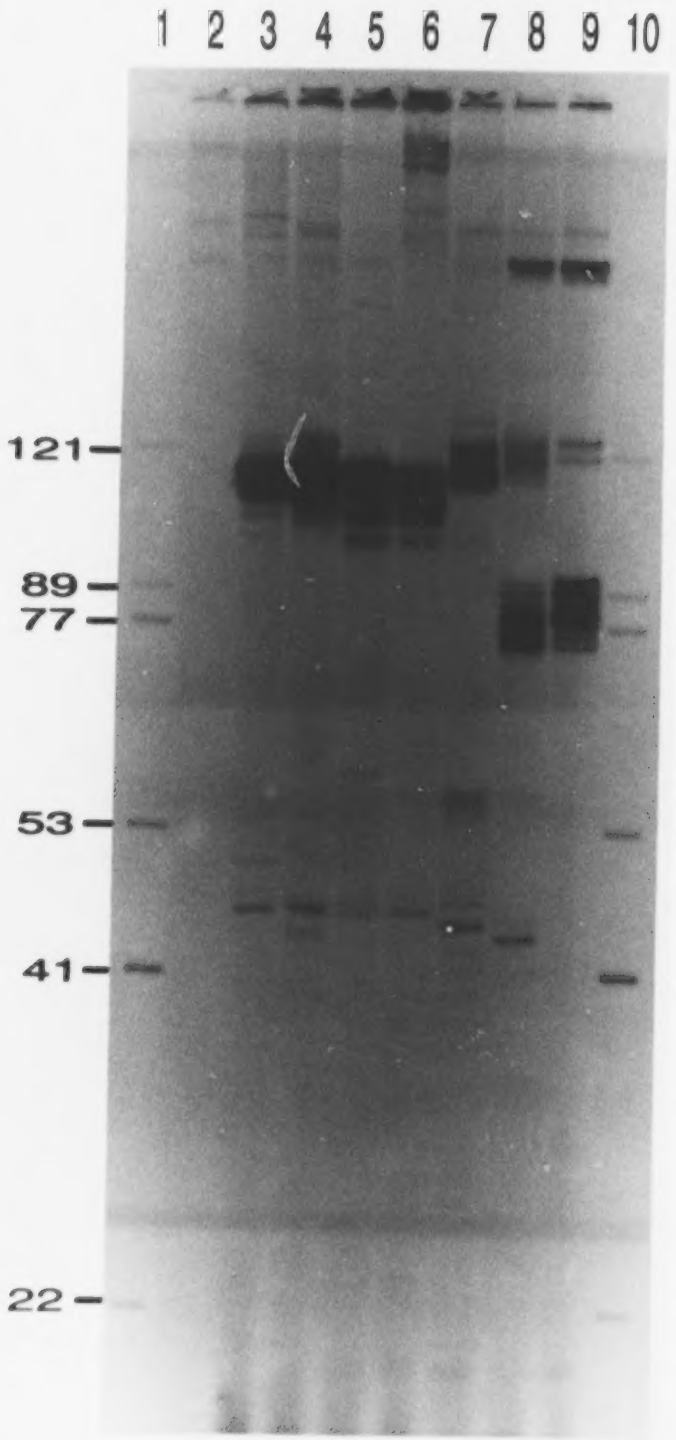

1 2 3 4 5 6 7 8 9 10
121
89
77
53
41
22

Figure 32. RNA transcripts from *in vitro* transcription reactions directed by recombinant plasmids containing the 6-1 $tRNA^{Tyr}$ gene.

The nucleic acids extracted from these *in vitro* reactions were separated electrophoretically on 10% denaturing polyacrylamide gels and the RNA transcripts synthesized *in vitro* were visualized by 8 - 16 hours of autoradiography at -20°C. Lane 1 contain single-stranded, radioactively labeled size markers which consisted of a mixture of 5S RNA from *E. coli* (121 nt.), $tRNA^{Phe}$ from brewer's yeast (77 nt.), and four synthetic oligonucleotides (89, 53, 41 and 22 nt.). Lane 2 is a control lane, showing the synthesis of products directed by the vector pUC118 alone. The recombinant plasmids directing the *in vitro* synthesis in lanes 3, - 6 were pM6128, pM6128-HB, pM6128-S and pM6128 #272, respectively.

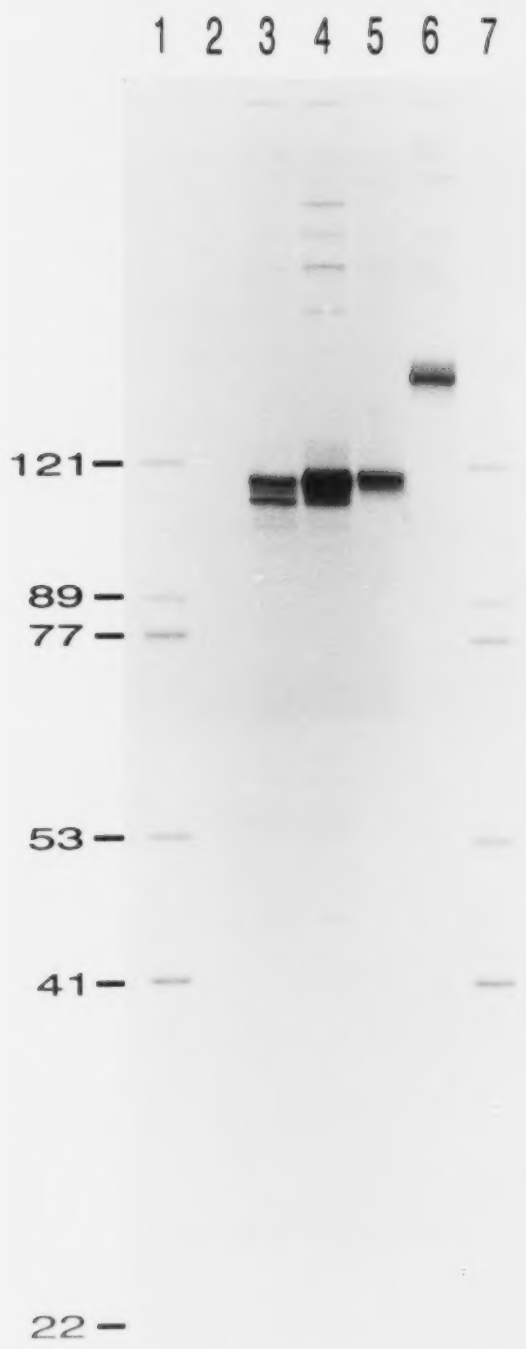

1 2 3 4 5 6 7
121
89
77
53
41
22

Figure 33. RNA transcripts from *in vitro* transcription reactions directed by recombinant plasmids containing the 6-2 $tRNA^{Tyr}$ gene.

The nucleic acids extracted from these *in vitro* reactions were separated electrophoretically on 10% denaturing polyacrylamide gels and the RNA transcripts synthesized *in vitro* were visualized by 8 - 16 hours of autoradiography at -20°C. Lane 1 contain single-stranded, radioactively labeled size markers which consisted of a mixture of 5S RNA from *E. coli* (121 nt.), $tRNA^{Phe}$ from brewer's yeast (77 nt.), and four synthetic oligonucleotides (89, 53, 41 and 22 nt.). Lane 2 is a control lane, showing the synthesis of products directed by the vector pUC118 alone. The recombinant plasmids directing the *in vitro* synthesis in lanes 3 and 4 are pM6 and pM6 #436, respectively.

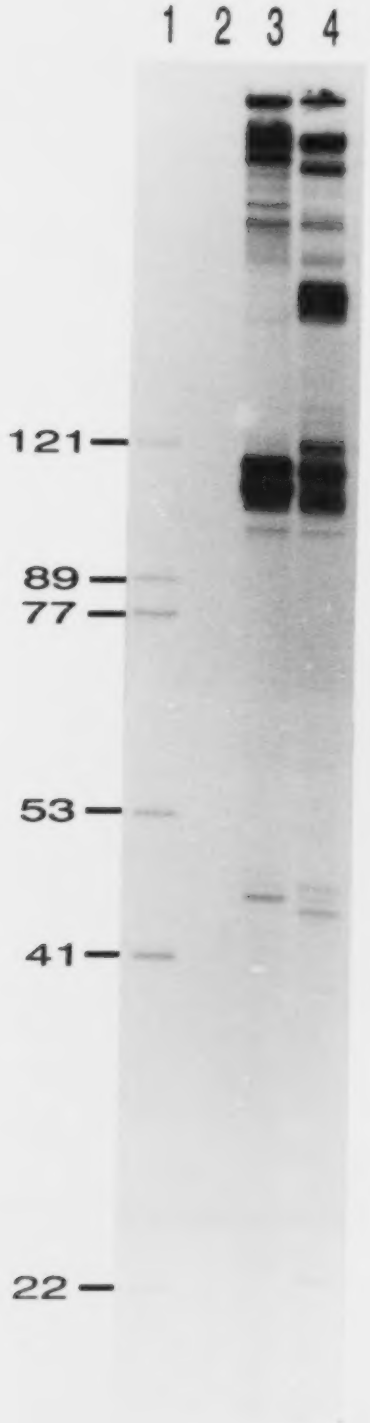

1 2 3 4
121
89
77
53
41
22

Figure 34. RNA transcripts from *in vitro* transcription reactions directed by recombinant plasmids containing the 6-3 $tRNA^{Tyr}$ gene.

The nucleic acids extracted from these *in vitro* reactions were separated electrophoretically on 10% denaturing polyacrylamide gels and the RNA transcripts synthesized *in vitro* were visualized by 8 - 16 hours of autoradiography at -20°C. Lane 1 contains single-stranded, radioactively labeled size markers which consisted of a mixture of 5S RNA from *E. coli* (121 nt.), $tRNA^{Phe}$ from brewer's yeast (77 nt.), and four synthetic oligonucleotides (89, 53, 41 and 22 nt.). Lane 2 is a control lane, showing the synthesis of products directed by the vector pUC118 alone. The recombinant plasmids directing the *in vitro* synthesis in lanes 3 - 6 are pM612, pM612-S, pM612-N and pM612 #50, respectively.

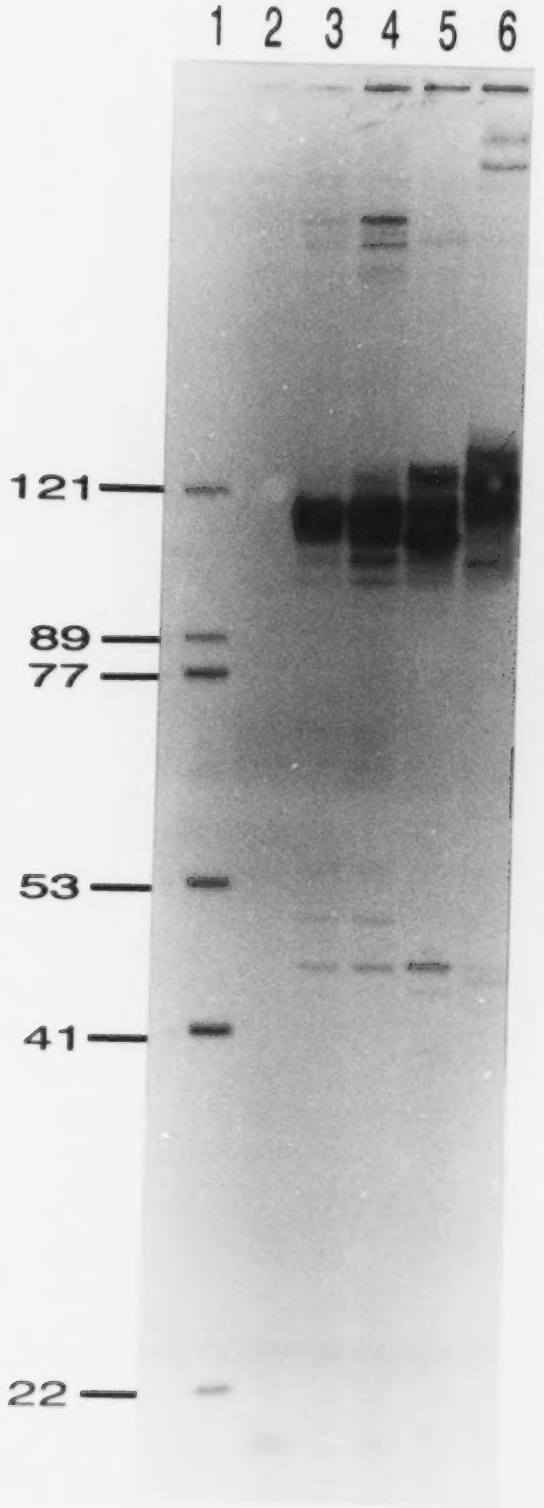

1 2 3 4 5 6
121
89
77
53
41
22

Figure 35. RNA transcripts from *in vitro* transcription reactions directed by recombinant plasmids containing the 6-4 $tRNA^{Tyr}$ gene.

The nucleic acids extracted from these *in vitro* reactions were separated electrophoretically on 10% denaturing polyacrylamide gels and the RNA transcripts synthesized *in vitro* were visualized by 8 - 16 hours of autoradiography at -20°C. Lane 1 contains single-stranded, radioactively labeled size markers which consisted of a mixture of 5S RNA from *E. coli* (121 nt.), $tRNA^{Phe}$ from brewer's yeast (77 nt.), and four synthetic oligonucleotides (89, 53, 41 and 22 nt.). Lane 2 is a control lane, showing the synthesis of products directed by the vector pUC118 alone. The recombinant plasmids directing the *in vitro* synthesis in lanes 3 - 7 are pM6IT, pM6IT-E, pM6IT-HE, pM6IT #39 and pM6IT #55, respectively.

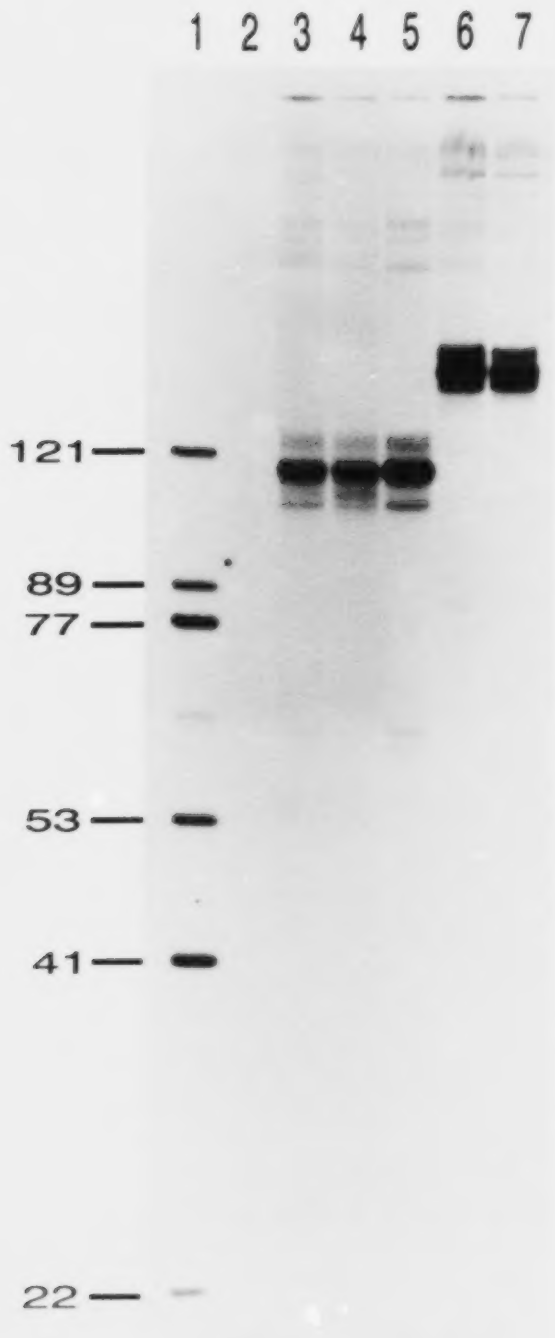

1 2 3 4 5 6 7
121
89
77
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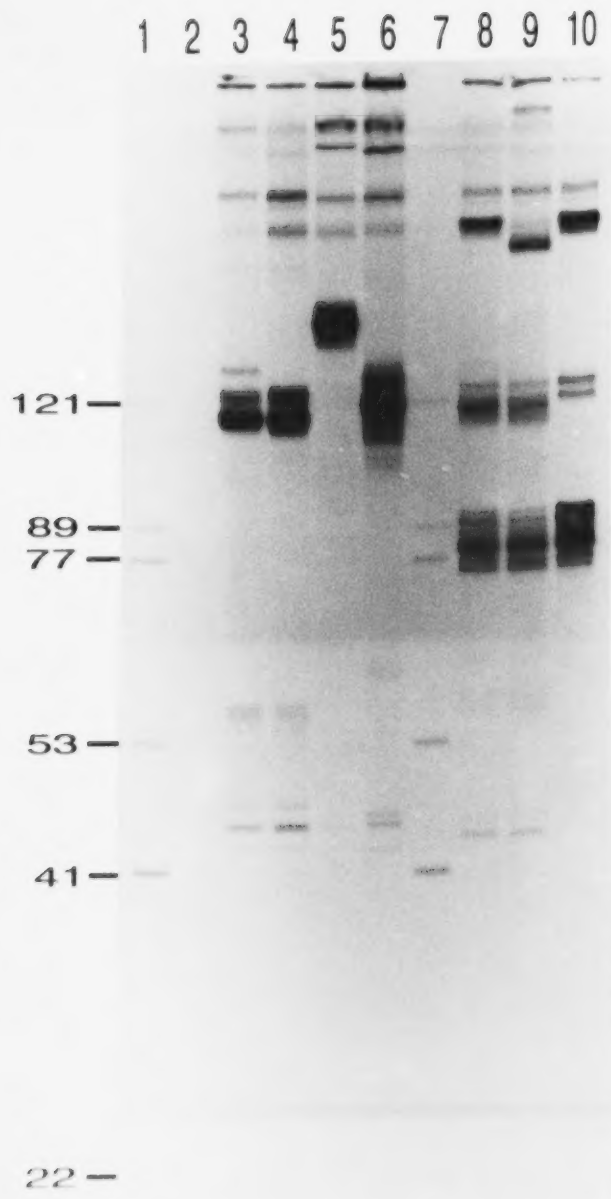
Figure 36. RNA transcripts from *in vitro* transcription reactions directed by recombinant plasmids containing tRNA genes from λHtM4.

The nucleic acids extracted from these *in vitro* reactions were separated electrophoretically on 10% denaturing polyacrylamide gels and the RNA transcripts synthesized *in vitro* were visualized by 8 - 16 hours of autoradiography at -20°C. Lanes 1 and 7 contain single-stranded, radioactively labeled size markers which consisted of a mixture of 5S RNA from *E. coli* (121 nt.), $tRNA^{Phe}$ from brewer's yeast (77 nt.), and four synthetic oligonucleotides (89, 53, 41 and 22 nt.). Lane 2 is a control lane, showing the synthesis of products directed by the vector pUC118 alone. The recombinant plasmids directing the *in vitro* synthesis in lanes 3 - 6 and 8 -10 are pJM4-HA, pJM4-MA, pJM4 #60, pJM4 #527, pJMS42-AE, pJMS42-SE and pDSALA.


1 2 3 4 5 6 7 8 9 10
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nt). However the RNA processing intermediates from each tRNA gene were distinct, since the major $tRNA^{Ala}$ intermediate was 76 nt and the $tRNA^{Tyr}$ intermediates were 42 and 53 nt (Figure 31, lane 8; Figure 36, lanes 8 and 9).

The identification of extragenic sequences capable of modulating tRNA gene expression was attempted by obtaining gene-containing plasmid clones with varying amounts of native flanking sequence and testing the ability of these recombinant plasmids to direct tRNA synthesis *in vitro*. The transcription efficiencies of the pM6128, pM6, pM612, pM6IT, and both pJM4-HA and pJMS42 $tRNA^{Tyr}$ gene-containing plasmids are compared in Figures 32, 33, 34, 35 and 36, respectively. All of these tRNA gene-containing plasmids were capable of directing the *in vitro* synthesis of pre-tRNA transcripts regardless of the sequences flanking the $tRNA^{Tyr}$ gene (Figures 31 - 36). However, some of the plasmid constructs that lacked native flanking sequences directed the synthesis of longer transcripts. The plasmid constructs that directed the synthesis of these longer transcripts were pM6128 #272 (Figure 32, lane 6), pM6IT #39 and #55 (Figure 35, lanes 6 and 7), and pJM4 #60 (Figure 36, lane 5). The recombinant plasmid pM6 #436 directed the synthesis of longer RNA transcripts due to the head-to-tail arrangement of the two 6-2 $tRNA^{Tyr}$ genes it harbours (Figure 33, lane 4).

The inability of the 5' flanking sequence deletions to cause changes in the transcription efficiencies, and the similar transcription activities of the $tRNA^{Tyr}$ genes, does not permit the identification of extragenic regulatory sequences. While there was some variation in tRNA transcription efficiencies from experiment to experiment, there were no consistent differences observed.

3.5 Self-cleavage of pre-tRNA

With the appearance of a report by van Tol *et al.* (1989) claiming that human precursor $tRNA^{Tyr}$ transcripts, from a gene identical to 4-1, can catalyze the excision of their introns, experiments were designed to reproduce and to extend their results. The

pre-tRNA^{Tyr} transcripts assayed for self-cleavage activity were generated by *in vitro* transcription with either mammalian cell extracts or T7 RNA polymerase. It was reasoned that if the pre-tRNA^{Tyr} transcripts have catalytic activity it should be retained regardless of the RNA polymerase that transcribes the tDNA sequence. All of the RNA self-cleavage assays were performed with radioactively labeled RNAs to allow the visualization of cleavage products by autoradiography. The experiments consisted of incubating gel-purified pre-tRNA^{Tyr} in 100 mM NH_4OAc (pH 8.0), 10 mM $MgCl_2$, 0.5 mM spermine and 0.4% Triton X-100 for a minimum of 2 hours at temperatures ranging from 37 to 46° C, the same conditions that had been used by van Tol *et al.* (1989). The pre-tRNA^{Tyr} transcripts synthesized by T7 RNA polymerase would also be free of any post-transcriptional modifications that might occur in eukaryotic systems. Therefore, the T7 generated transcripts would serve as controls to test whether the RNA self-cleavage is a consequence of post-transcriptional events (e.g. methylation of the pre-tRNA) that occur in mammalian cell extracts.

To obtain pre-tRNA^{Tyr} transcripts synthesized by T7 RNA polymerase the tDNA sequences were amplified by PCR and cloned into pBS, which has a T7 promoter adjacent to the multiple cloning site. These T7 generated transcripts, while free of any eukaryotic post-transcriptional modifications, did not have native 5' leader and 3' trailer sequences. T7 RNA polymerase initiated transcription from its promoter and transcribed both vector and tDNA sequences until it reached the end of the linearized DNA template. The resulting pre-tRNA^{Tyr} transcripts were chimeric, with the 5' leader and 3' trailer sequences a consequence of the vector DNA flanking the cloned tRNA^{Tyr} genes. Another factor that made the T7 pre-tRNA^{Tyr} transcripts chimeric was the mutation(s) introduced into the PCR amplified tDNA sequences as a result of degenerate positions on one of the oligonucleotide primers (i.e. DSP 2).

In order to distinguish RNA self-cleavage from degradation by ribonuclease contamination, three RNAs (*E. coli* 5S rRNA, yeast tRNA^{Phe} and human pre-tRNA^{Ala})

served as controls. These RNAs were chosen as controls because they do not contain introns and they were available in sufficient quantities. The control RNAs were assayed alongside the pre-tRNA^{Tyr} transcripts and did not usually show any self-cleavage or degradation, except for the human pre-tRNA^{Ala} transcripts, which yielded cleavage products when the incubation temperature was increased to 46°C. Occasional ribonuclease contamination (e.g. lane 11 of Figure 38, lanes 2 and 11 of Figure 39, and lane 10 of Figure 41) of the RNA self-cleavage assay did occur, which was evident from the unusually high amount of RNA degradation products visualized.

Initially, the self-cleavage assays were performed at 37°C for 2 hours with pre-tRNA^{Tyr} transcripts synthesized *in vitro* with 293 S-100 cell extracts. Under these conditions the pre-tRNA^{Tyr} transcripts exhibited very little self-cleavage activity, which required three days of autoradiography at -80°C to detect. To increase the amount of RNA cleavage the experiments were repeated with the temperature increased from 37 to 42°C and the length of the incubation also increased slightly from 2 to 2.5 hours. The higher temperature resulted in increased amounts of pre-tRNA^{Tyr} cleavage products, which were detected by 18 hours of autoradiography at -80°C. With the assay conditions modified for increased RNA cleavage activity, pre-tRNA^{Tyr} transcripts synthesized by T7 RNA polymerase and 293 S-100 cell extracts were assayed for self-cleavage.

All of the pre-tRNA^{Tyr} transcripts were capable of self-cleavage, regardless of their origins. Figure 37 shows that self-cleavage of 4-1 pre-tRNA^{Tyr} transcripts, whether synthesized with 293 cell extracts of T7 RNA polymerase (lanes 3 and 12, respectively), generated two major products and several minor species ranging in size from 50 to 70 nt. The pre-tRNA^{Tyr} transcripts (112 to approximately 135 nt) yielded cleavage products, similar in size to the RNA processing intermediates generated during *in vitro* transcription assays, that ranged in size from 50 to 70 nt.

While cleavage products derived from the pre-tRNA^{Tyr} transcripts were observed by autoradiography it was still not certain whether the cleavage patterns were due to RNA

Figure 37. Autoradiograph of the 4-1 pre-tRNA^{Tyr} transcripts and their cleavage products.

The pre-tRNA transcripts and their cleavage products were fractionated on 10% denaturing polyacrylamide gels and visualized by autoradiography. RNA self-cleavage assays were performed at 42°C for 2.5 hours with 4-1 pre-tRNA^{Tyr} (lanes 2 - 9, 11 - 14 and 4-3 pre-tRNA^{Ala} (lanes 15 -18) transcripts. The DNA templates, combined with 293 S-100 cell extracts that directed the *in vitro* synthesis of the transcripts in lanes 2 - 5, lanes 6 - 9, and lanes 15 - 18, were pJM4-HA, pJM4 #60 and pDSALA respectively. The transcripts in lanes 11 -14 were synthesized with T7 RNA polymerase using linearized pJM4 #60 template. Lanes 1, 10 and 19 contain radioactively labeled single-stranded size markers. Lanes 2, 6, 11 and 15 contain samples of pre-tRNA transcripts after extraction from polyacrylamide gels. The pre-tRNA transcripts in lanes 3, 7, 12 and 16 have been assayed in the absence of oligonucleotides. The pre-tRNA transcripts in lanes 4, 8, 13 and 17 have been assayed for self-cleavage in the presence of 1 pmol of RWH 61, an oligonucleotide complementary to the 4-1 pre-tRNA^{Tyr} intron. The pre-tRNA transcripts in lanes 5, 9, 14 and 18 have been assayed in the presence of 10 pmol of RWH 61.

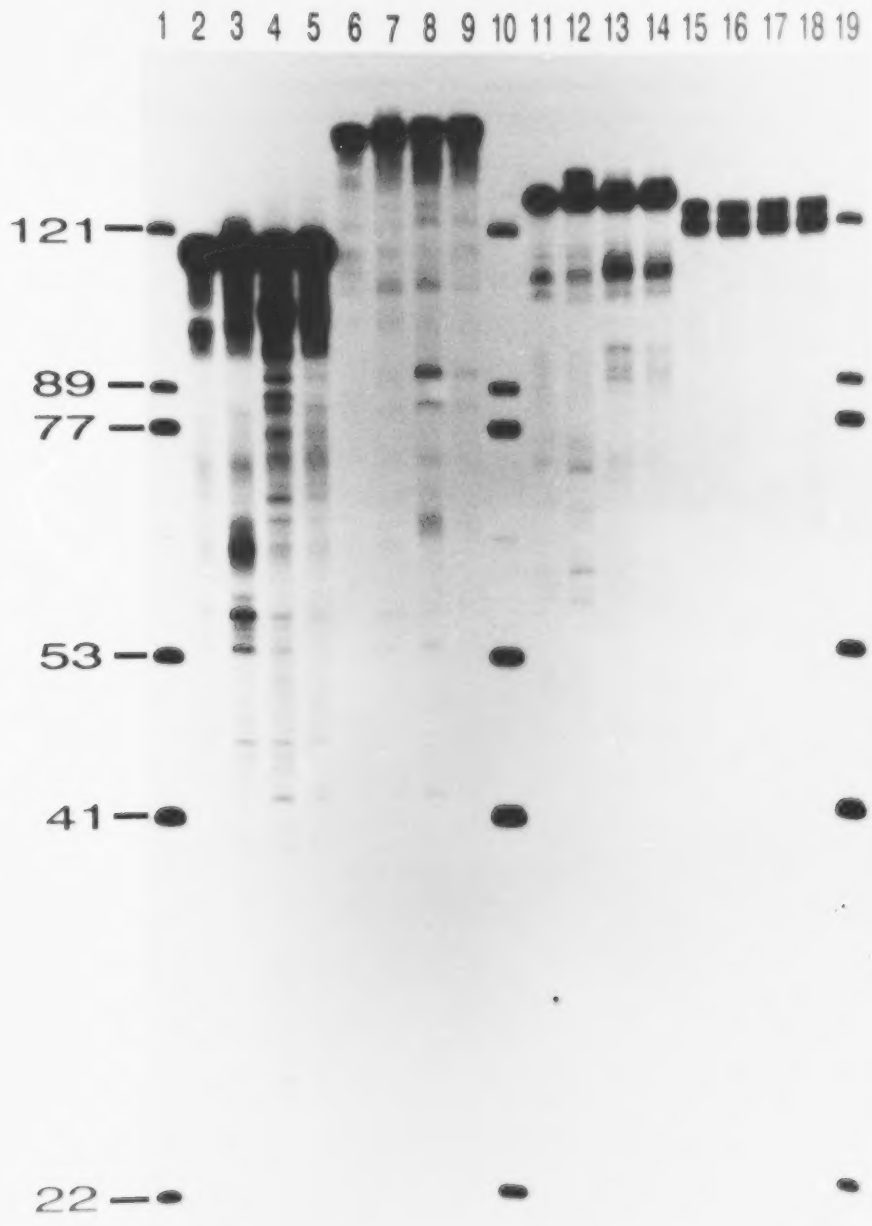
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
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Figure 38. Autoradiograph of the 4-1, 6-2 and 6-3 pre-tRNA^{Tyr} transcripts and their cleavage products.

The pre-tRNA transcripts and their cleavage products were fractionated on 10% denaturing polyacrylamide gels and visualized by autoradiography. The RNA self-cleavage assays were performed at 42°C for 2.5 hours. The linearized DNA templates that directed T7 RNA polymerase to synthesize the transcripts assayed in lanes 2 - 6, 8 - 12 and 14 - 18, were pJM4 #60, pM6 #436 and pM612 #50 respectively. Lanes 1, 7, 13 and 19 contain radioactively labeled single-stranded size markers. Lanes 2, 8 and 14 contain untreated transcripts that served as controls. The pre-tRNA transcripts in lanes 3, 9 and 15 have been assayed for self-cleavage in the absence of oligonucleotides. The pre-tRNA transcripts in lanes 4, 10 and 16 have been assayed with 10 pmol of RWH 61, an oligonucleotide complementary to the 4-1 pre-tRNA^{Tyr} intron. The pre-tRNA transcripts in lanes 5, 11 and 17 have been assayed with 10 pmol of DSP 2, an oligonucleotide complementary to the 3' half of tRNA^{Tyr}. The pre-tRNA transcripts in lanes 6, 12 and 18 have been assayed with 10 pmol of DSP 1, an oligonucleotide identical in sequence to the 5' half of tRNA^{Tyr}.

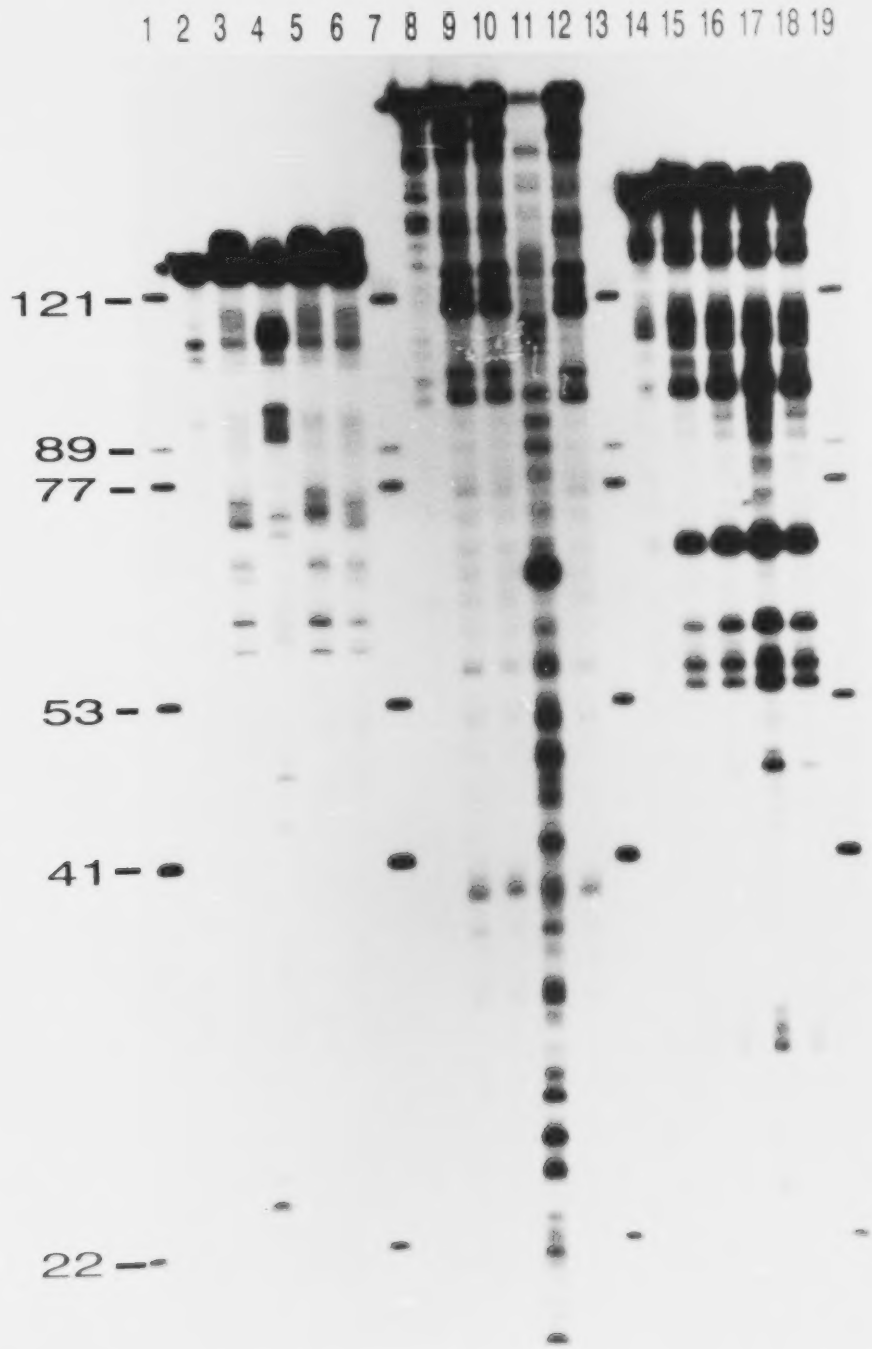
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
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Figure 39. Autoradiograph of the 4-1, 6-4 and 6-1 pre-tRNA^{Tyr} transcripts and their cleavage products.

The pre-tRNA transcripts and their cleavage products were fractionated on 10% denaturing polyacrylamide gels and visualized by autoradiography. The RNA self-cleavage assays were performed at 42°C for 2.5 hours. The linearized DNA templates that directed T7 RNA polymerase to synthesize the transcripts assayed in lanes 2 - 6, 8 - 12 and 14 - 18, were pJM4 #527, pM6 #55 and pM6128 #272 respectively. Lanes 1, 7 and 13 contain radioactively labeled single-stranded size markers. Lanes 2, 8 and 14 contained untreated transcripts that served as controls. The pre-tRNA transcripts in lanes 3, 9 and 15 have been assayed in the absence of oligonucleotides. The pre-tRNA transcripts in lanes 4, 10 and 16 have been assayed with 10 pmol of RWH 61, an oligonucleotide complementary to the 4-1 pre-tRNA^{Tyr} intron. The pre-tRNA transcripts in lanes 5, 11 and 17 have been assayed with 10 pmol of DSP 2, an oligonucleotide complementary to the 3' half of tRNA^{Tyr}. The pre-tRNA transcripts in lanes 6, 12 and 18 have been assayed with 10 pmol of DSP 1, an oligonucleotide identical in sequence to the 5' half of tRNA^{Tyr}.

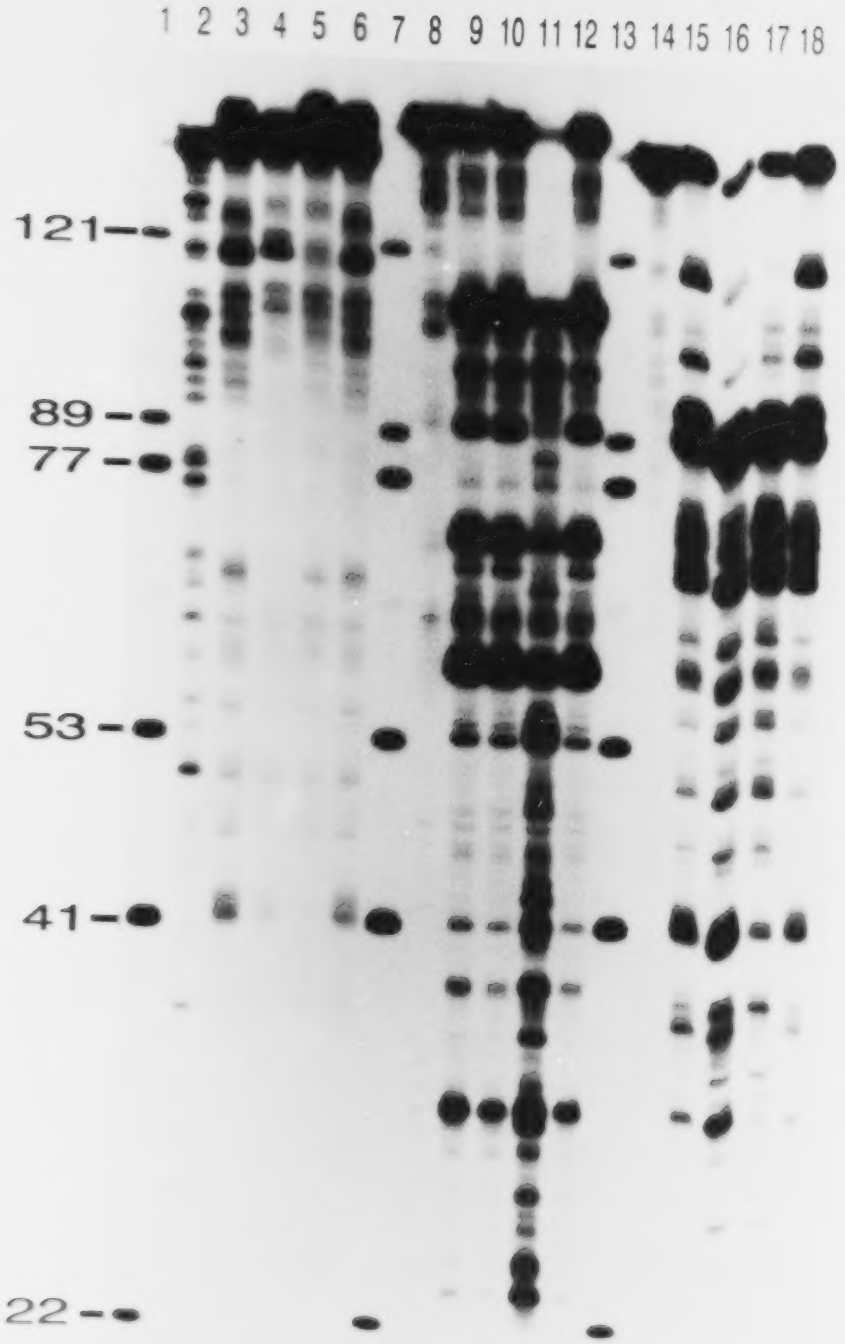
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
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Figure 40. Autoradiograph of the 6-1 and 6-4 pre-tRNA^{Tyr} transcripts and their cleavage products.

The pre-tRNA transcripts and their cleavage products were fractionated on 10% denaturing polyacrylamide gels and visualized by autoradiography. The RNA self-cleavage assays were performed at 46°C for 2.5 hours. The self-cleavage assays in lanes 2 - 10 and in lanes 12 - 20 were performed with 6-1 and 6-4 pre-tRNA^{Tyr} transcripts respectively, synthesized with 293 S-100 cell extracts using recombinant plasmids pM6128 and pM6IT. Lanes 1 and 11 contain radioactively labeled single-stranded size markers. Lanes 2 and 12 are controls which contain untreated pre-tRNA transcripts. The pre-tRNA transcripts in lanes 3 and 13 have been assayed in the absence of oligonucleotides. The 6-1 pre-tRNA^{Tyr} transcripts in lanes 4 - 10 have been assayed in the presence of 50 pmol of oligonucleotides RWH 61, DSP 19, DSP 20, DSP 21, DSP 22, DSP 23 and DSP 2, respectively. The 6-4 pre-tRNA^{Tyr} transcripts in lanes 14 - 20 have also been assayed in the presence of 50 pmol of oligonucleotides RWH 61, DSP 19, DSP 20, DSP 21, DSP 22, DSP 23 and DSP 2, respectively. The oligonucleotides RWH 61, DSP 19, DSP 20, DSP 21 and DSP 22 are complementary to the intron sequences of the 4-1, 6-2, 6-1, 6-3 and 6-4 tRNA^{Tyr} genes, respectively. The oligonucleotide DSP 23 is complementary to the 5' half of tRNA^{Tyr}, while oligonucleotide DSP 2 is complementary to the 3' half of tRNA^{Tyr}.

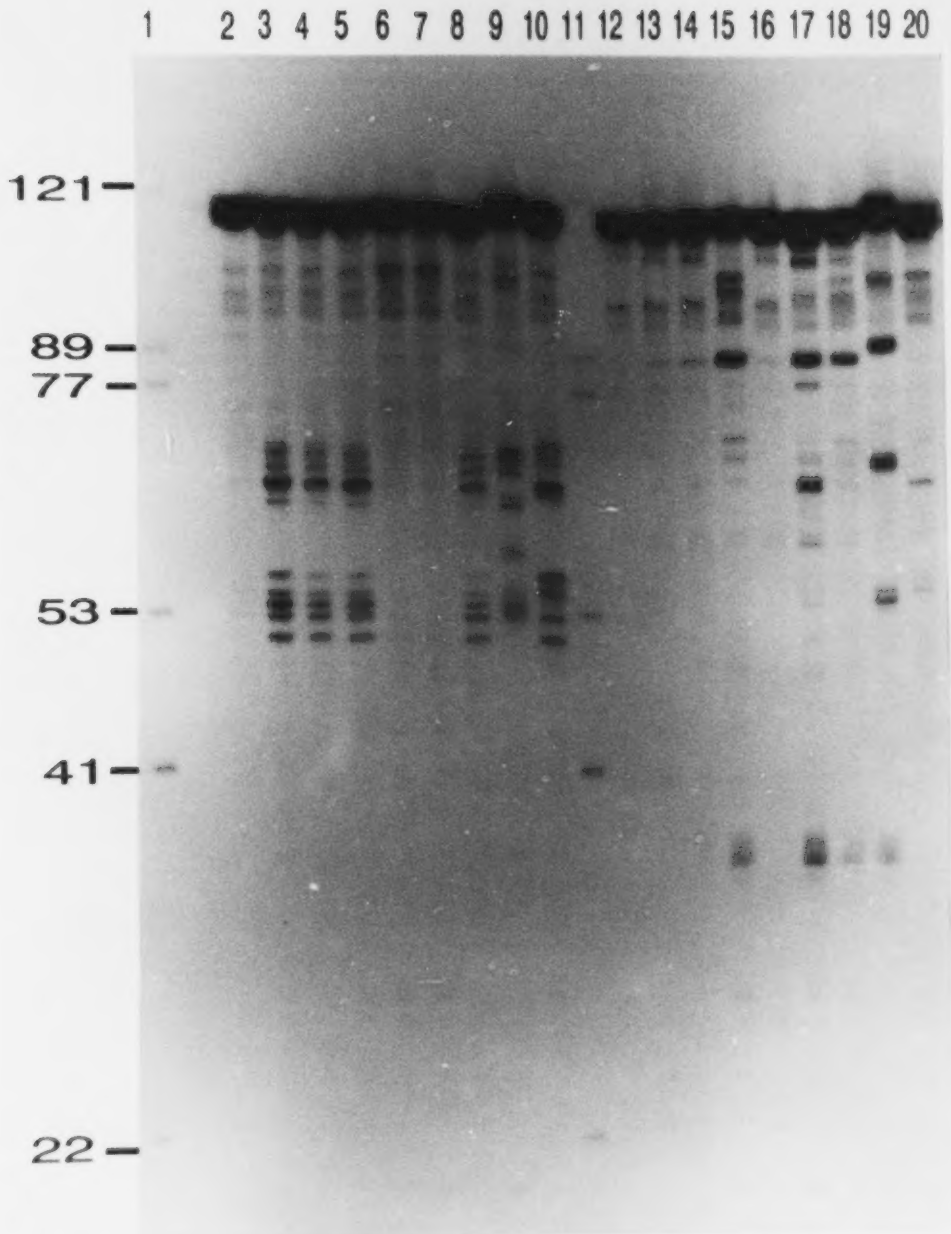
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
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Figure 41. Autoradiograph of the 6-3 and 6-2 pre-tRNA^{Tyr} transcripts and their cleavage products.

The pre-tRNA transcripts and their cleavage products were fractionated on 10% denaturing polyacrylamide gels and visualized by autoradiography. The RNA self-cleavage assays were performed at 46°C for 2.5 hours. The self-cleavage assays in lanes 3 - 10 and in lanes 13 - 20 were performed with 6-3 and 6-2 pre-tRNA^{Tyr} transcripts respectively, synthesized with 293 S-100 cell extracts using recombinant plasmids pM612 and pM6. Lanes 1 and 11 contain radioactively labeled single-stranded size markers. Lanes 2 and 13 are controls which contain untreated pre-tRNA transcripts. The pre-tRNA transcripts in lanes 3 and 13 have been assayed in the absence of oligonucleotides. The 6-3 pre-tRNA^{Tyr} transcripts in lanes 4 - 10 have been assayed in the presence of 50 pmol of oligonucleotides RWH 61, DSP 19, DSP 20, DSP 21, DSP 22, DSP 23 and DSP 2, respectively. The 6-2 pre-tRNA^{Tyr} transcripts in lanes 14 - 20 have also been assayed in the presence of 50 pmol of oligonucleotides RWH 61, DSP 19, DSP 20, DSP 21, DSP 22, DSP 23 and DSP 2, respectively. The oligonucleotides RWH 61, DSP 19, DSP 20, DSP 21 and DSP 22 are complementary to the intron sequences of the 4-1, 6-2, 6-1, 6-3 and 6-4 tRNA^{Tyr} genes, respectively. The oligonucleotide DSP 23 is complementary to the 5' half of tRNA^{Tyr}, while oligonucleotide DSP 2 is complementary to the 3' half of tRNA^{Tyr}.

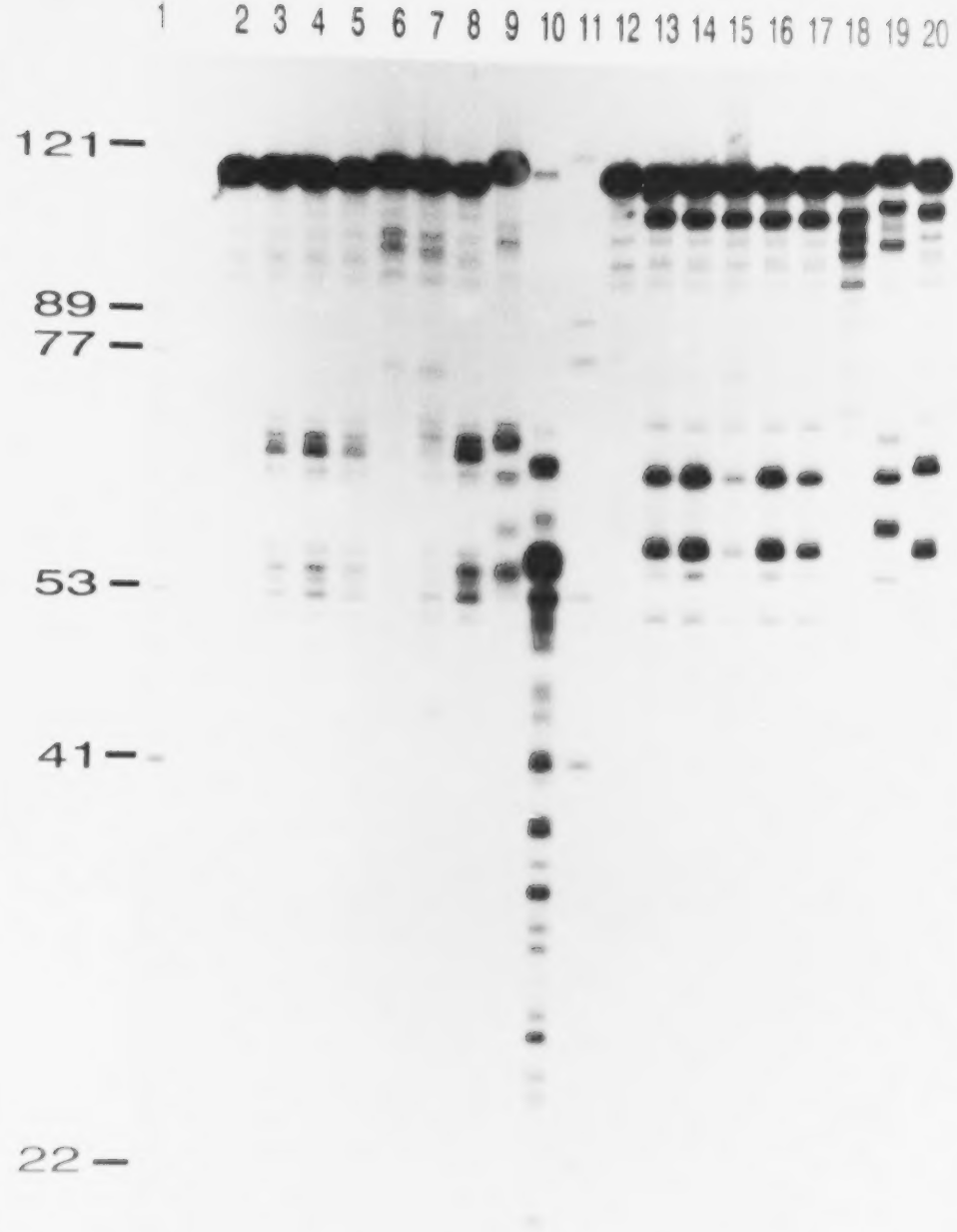
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
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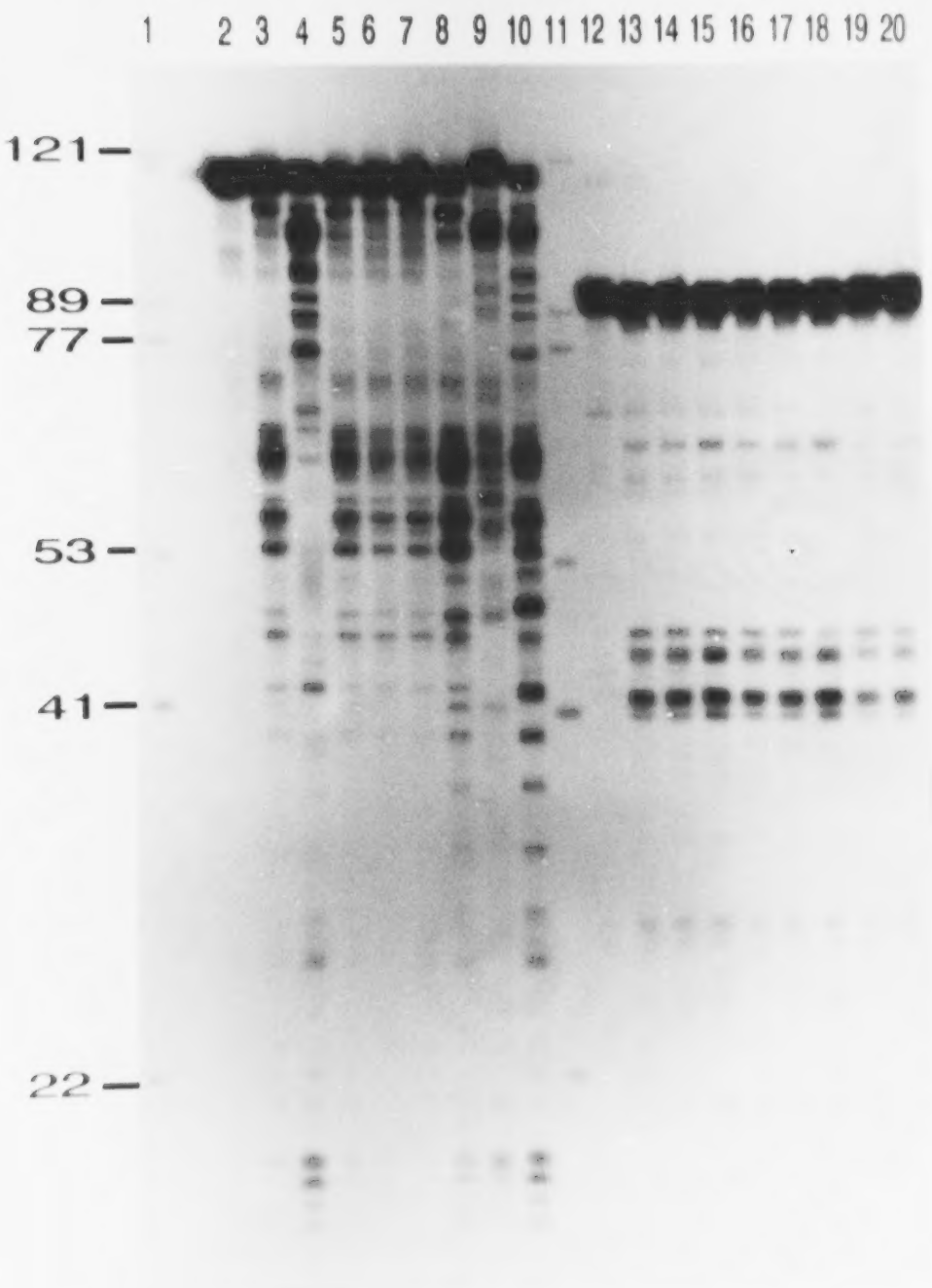
Figure 42. Autoradiograph of the 4-1 pre-tRNA^{Tyr} and the 4-3 pre-tRNA^{Ala} transcripts and their cleavage products.

The pre-tRNA transcripts and their cleavage products were fractionated on 10% denaturing polyacrylamide gels and visualized by autoradiography. The RNA self-cleavage assays were performed at 46°C for 2.5 hours. The self-cleavage assays in lanes 2 - 10 and in lanes 12 - 20 were performed with 4-1 pre-tRNA^{Tyr} and 4-3 pre-tRNA^{Ala} transcripts respectively, synthesized with 293 S-100 cell extracts using recombinant plasmids pJM4-HA and pDSALA. Lanes 1 and 11 contain radioactively labeled single-stranded size markers. Lanes 2 and 12 are controls which contain untreated pre-tRNA transcripts. The pre-tRNA transcripts in lanes 3 and 13 have been assayed in the absence of oligonucleotides. The 4-1 pre-tRNA^{Tyr} transcripts in lanes 4 - 10 have been assayed in the presence of 50 pmol of oligonucleotides RWH 61, DSP 19, DSP 20, DSP 21, DSP 22, DSP 23 and DSP 2, respectively. The 4-3 pre-tRNA^{Ala} transcripts in lanes 14 - 20 have also been assayed in the presence of 50 pmol of oligonucleotides RWH 61, DSP 19, DSP 20, DSP 21, DSP 22, DSP 23 and DSP 2, respectively. The oligonucleotides RWH 61, DSP 19, DSP 20, DSP 21 and DSP 22 are complementary to the intron sequences of the 4-1, 6-2, 6-1, 6-3 and 6-4 tRNA^{Tyr} genes, respectively. The oligonucleotide DSP 23 is complementary to the 5' half of tRNA^{Tyr}, while oligonucleotide DSP 2 is complementary to the 3' half of tRNA^{Tyr}.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
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autocatalysis. In an attempt to resolve this problem, oligonucleotides, directed at either the exon or intron portions of the pre-tRNA^{Tyr} transcripts, were included in the RNA cleavage assays. If the RNA cleavage patterns were due to inherent lability of the RNA at certain positions in the transcript, it was hypothesized that the addition of antisense or sense oligonucleotides should not modify the RNA cleavage patterns. However, if the pre-tRNA^{Tyr} transcripts are autocatalytic, then perturbation of their secondary and tertiary structure by the annealing of oligonucleotides should result in a loss of catalytic activity.

The addition of antisense oligonucleotides directed toward the introns of the pre-tRNA^{Tyr} transcripts had an inhibitory effect on RNA self-cleavage, which seems indicative of RNA self-cleavage by RNA autocatalysis. Increasing the amounts of antisense oligonucleotide in the RNA cleavage assay also increased the amount of inhibition observed (Figure 37). Inhibition of RNA self-cleavage was observed, regardless of the origin of the pre-tRNA^{Tyr} transcripts, whenever an antisense oligonucleotide was capable of annealing to the transcript's intervening sequence (Figures 37 - 42).

The incubation temperature for the RNA self-cleavage assay was raised from 42° to 46°C to promote DNA-RNA duplex formation by reducing the tertiary and secondary structures of the pre-tRNA transcripts. The RNA cleavage assays performed under these conditions also resulted in specific cleavage patterns (Figures 40 - 42). Inhibition of RNA cleavage occurred when antisense oligonucleotides designed for each pre-tRNA^{Tyr} intron could anneal to the pre-tRNA in the assay (e.g. Figure 37, lane 5; Figure 40, lanes 6 and 7; Figure 41, lane 18). However, antisense and sense oligonucleotides designed for the pre-tRNA^{Tyr} exons only caused changes in the cleavage patterns on occasion (Figures 40 - 42). The change in temperature from 42° to 46°C led to self-cleavage of the 89 nt pre-tRNA^{Ala}, whose cleavage was not inhibited by any of the oligonucleotides designed for the pre-tRNA^{Tyr} transcripts (Figure 42, lanes 13 - 20). The 121 nt pre-tRNA^{Ala} did not undergo self-cleavage when the assay was performed at 42°C (Figure 37, lanes 16 - 18). When 6-4 pre-tRNA^{Tyr} transcripts were assayed for self-cleavage in the presence of

antisense oligonucleotides, designed for the intron sequences, cleavage was promoted rather than inhibited (Figure 40). Further self-cleavage assays with 6-4 pre-tRNA^{Tyr} transcripts are required to ensure these anomalous results are reproducible.

While it appears that RNA self-cleavage, catalyzed by magnesium ions, has occurred, it is not certain if it has released the intron. The cleavage products will have to be sequenced, or the cleavage sites mapped, to determine if intron excision has occurred.

4. Discussion

Three human-λ recombinants carrying tyrosine tRNA genes were previously isolated from a human-λ Charon-4A recombinant phage library using a probe which contains a $tRNA^{Tyr}$ gene derived from cloned *Xenopus laevis* DNA (Müller and Clarkson, 1980). Initially, MacPherson (1988) detected six intron-containing tyrosine tRNA genes by Southern analysis, with four genes detected on λHtM6 and single genes detected on both λHtM2 and λHtM4. The nucleotide sequences of five $tRNA^{Tyr}$ genes and their flanking sequences were determined, however, the $tRNA^{Tyr}$ gene on λHtM2 remained uncharacterized. A portion of this study included determining the nucleotide sequence of an 855 bp *Hin*dIII/*Bgl*II fragment from λHtM2, which contained this uncharacterized $tRNA^{Tyr}$ gene. The nucleotide sequence of this intron-containing $tRNA^{Tyr}$ gene, tentatively named M2, and its flanking sequences were identical to those of the 6-1 $tRNA^{Tyr}$ gene found on λHtM6. The identical flanking sequences surrounding these two $tRNA^{Tyr}$ genes suggested that λHtM2 and λHtM6 could be overlapping bacteriophage clones. A Southern cross experiment between λHtM2 and λHtM6 provided additional evidence that they are in fact overlapping clones (Figure 6). Since the existing restriction endonuclease maps assembled by MacPherson (1988) did not show any common overlapping region, revisions to these restriction maps were necessary. However, complete restriction endonuclease digestions of λHtM2 and λHtM6 were found to be insufficient for generating unambiguous maps.

Restriction maps were finally constructed by combining data from complete restriction endonuclease digests with those of partial digests. Initially the restriction endonuclease mapping strategy described by Rackwitz *et al.* (1984) was followed. However, the length of the left and right arms of Charon 4A (19.9 and 11.0 kb, respectively) severely limited the accuracy of this method, which was dependent on the resolution of agarose gel electrophoresis. By removing vector sequences with restriction

enzymes that do not cut the insert, partial digests were performed on 20 to 25 kb DNA fragments instead of 45 to 50 kb recombinant bacteriophage. DNA fragments of 20 to 25 kb were partially digested, fractionated electrophoretically, transferred and probed sequentially with left and right arm probes to generate a restriction map for each enzyme chosen.

This modified strategy for restriction endonuclease mapping led to changes in the λHtM2, λHtM4 and λHtM6 restriction maps (Figure 16). The overlapping region between λHtM2 and λHtM6, that contains the 6-1 $tRNA^{Tyr}$ gene, is now evident on the revised restriction maps. Amendments to the restriction map of λHtM4 led to the serendipitous discovery of a previously undetected $tRNA^{Tyr}$ gene. Digestion of λHtM4 DNA with restriction endonuclease *Apa*I was performed to remove bacteriophage λ vector sequences prior to partial digestion; however, *Apa*I released two fragments that were found to hybridize with an oligonucleotide probe specific for $tRNA^{Tyr}$ genes (Figure 13). Since the putative $tRNA^{Tyr}$ gene was also contained on the λHtM4 subclone pJM4, the sequencing of the 2.4 kb insert was continued in order to characterize this new tRNA gene (Figure 20). The coding sequence of this new $tRNA^{Tyr}$ gene; named 4-2, was virtually identical to the other five human $tRNA^{Tyr}$ genes, however, its intervening sequence was markedly different from those of the other genes as indicated by its high GC content. While searching the nucleotide sequence of this 2.4 kb *Eco*RI/*Hin*dIII fragment from λHtM4 for the locations of the tyrosine tRNA genes, an alanine tRNA gene was also identified. This $tRNA^{Ala}$ gene was later proved to be a *bona fide* gene based on sequence comparisons with other $tRNA^{Ala}$ genes (Figure 43) and by *in vitro* expression experiments which have shown this gene to be transcriptionally active (Figures 31 and 36). The alanine gene that was isolated is identical to one of the $tRNA^{Ala}$ sequences determined by Bunn and Mathews (1987). They utilized antibodies against $tRNA^{Ala}$ found in the sera of patients suffering from polymyositis, an autoimmune disease, to immunoprecipitate sufficient quantities of tRNA for sequencing.

Figure 43. Comparison of the 4-3 $tRNA^{Ala}$ gene sequence with $tRNA^{Ala}$ gene sequences from the literature

The alignment was done on six $tRNA^{Ala}$ gene-containing sequences. The character to show that a position in the alignment is perfectly conserved is '*'. The character to show that a position is well conserved is '.'. The tDNA sequences are shown in bold letters. The sources of the sequences used for the alignment are listed below:

(1) human gene, 4-3

(2) *Drosophila* gene (Delotto and Schedl, 1984)

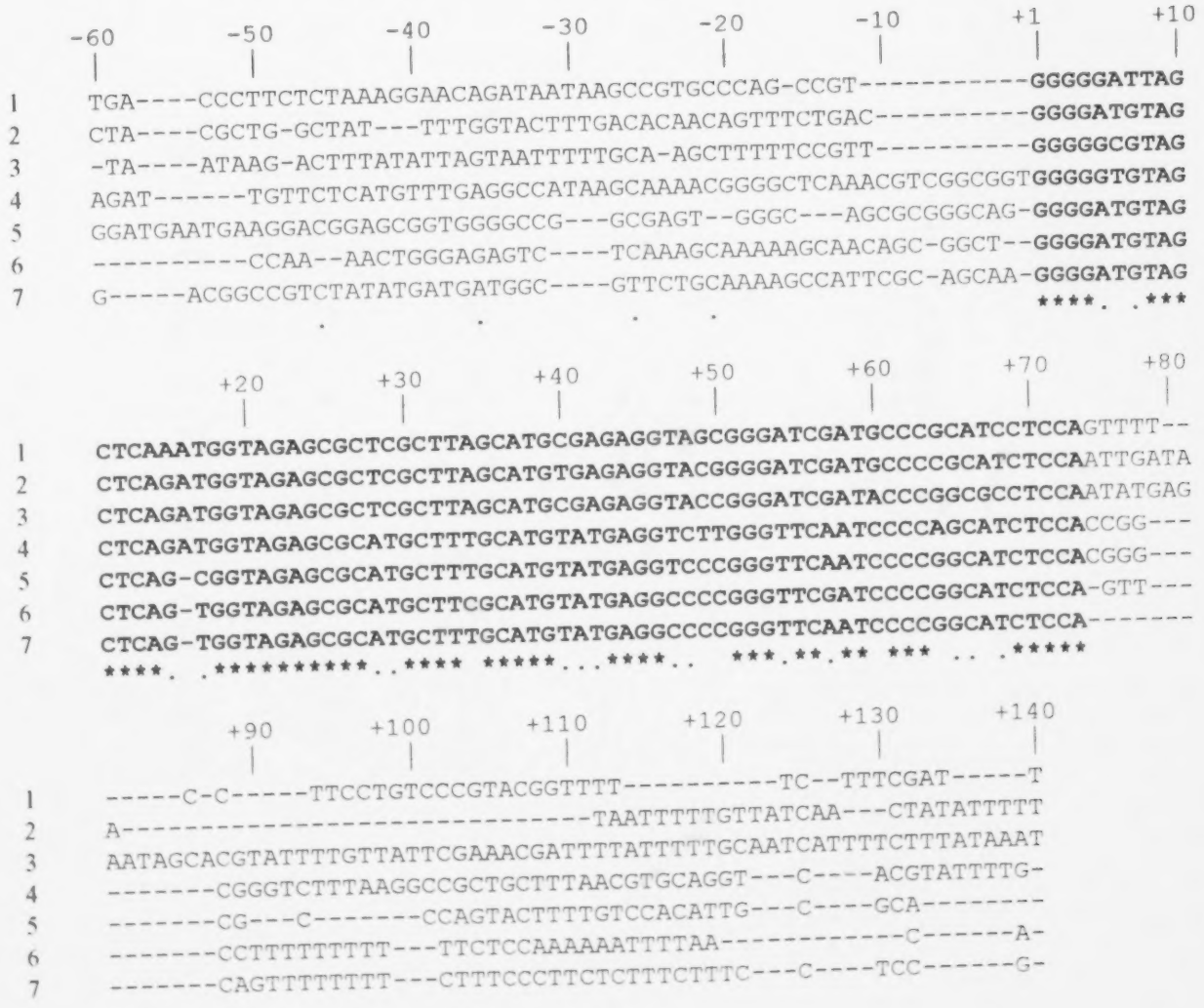
(3) *Bombyx* gene (Young et al., 1991)

(4) *Xenopus* gene (Müller et al., 1987)

(5) chicken gene (Mezquita and Mezquita, 1992)

(6) mouse gene (Russo et al., 1986)

(7) chicken gene (Mezquita and Mezquita, 1992).


-60 -50 -40 -30 -20 -10 +1 +10
1 TGA----CCCTTCTCTAAAGGAACAGATAATAAGCCGTGCCCAG-CCGT----------GGGGGATTAG
2 CTA----CGCTG-GCTAT---TTTGGTACTTTGACACAACAGTTTCTGAC----------GGGGATGTAG
3 -TA----ATAAG-ACTTTATATTAGTAATTTTTGCA-AGCTTTTTCCGTT----------GGGGGCGTAG
4 AGAT------TGTTCTCATGTTTGAGGCCATAAGCAAAACGGGGCTCAAACGTCGGCGGTGGGGGTGTAG
5 GGATGAATGAAGGACGGAGCGGTGGGGCCG---GCGAGT--GGGC---AGCGCGGGCAG-GGGGATGTAG
6 ----------CCAA--AACTGGGAGAGTC----TCAAAGCAAAAAGCAACAGC-GGCT--GGGGATGTAG
7 G-----ACGGCCGTCTATATGATGATGGC----GTTCTGCAAAAGCCATTCGC-AGCAA-GGGGATGTAG
**** . .***
+20 +30 +40 +50 +60 +70 +80
1 CTCAAATGGTAGAGCGCTCGCTTAGCATGCGAGAGGTAGCGGGATCGATGCCCGCATCCTCCAGTTTT--
2 CTCAGATGGTAGAGCGCTCGCTTAGCATGTGAGAGGTACGGGGATCGATGCCCCGCATCTCCAATTGATA
3 CTCAGATGGTAGAGCGCTCGCTTAGCATGCGAGAGGTACCGGGATCGATACCCGGCGCCTCCAATATGAG
4 CTCAGATGGTAGAGCGCATGCTTTGCATGTATGAGGTCTTGGGTTCAATCCCCAGCATCTCCACCGG---
5 CTCAG-CGGTAGAGCGCATGCTTTGCATGTATGAGGTCCCGGGTTCAATCCCCGGCATCTCCACGGG---
6 CTCAG-TGGTAGAGCGCATGCTTCGCATGTATGAGGCCCCGGGTTCGATCCCCGGCATCTCCA-GTT---
7 CTCAG-TGGTAGAGCGCATGCTTTGCATGTATGAGGCCCCGGGTTCAATCCCCGGCATCTCCA-------
**** . .********** ..**** ***** ...**** .. *** .** .** *** .. .*****
+90 +100 +110 +120 +130 +140
1 -----C-C-----TTCCTGTCCCGTACGGTTTT----------TC--TTTCGAT-----T
2 A----------TAATTTTTGTTATCAA---CTATATTTTT
3 AATAGCACGTATTTTGTTATTCGAAACGATTTTATTTTTGCAATCATTTTCTTTATAAAT
4 -------CGGGTCTTTAAGGCCGCTGCTTTAACGTGCAGGT---C----ACGTATTTTG-
5 -------CG---C-------CCAGTACTTTTGTCCACATTG---C----GCA--------
6 -------CCTTTTTTTTT---TTCTCCAAAAAATTTTAA----------C------A-
7 -------CAGTTTTTTTT---CTTTCCCTTCTCTTTCTTTC---C----TCC------G-

While the restriction endonuclease maps of the three bacteriophage recombinants allowed the gene-containing fragments to be positioned unambiguously, the orientations of some of these tRNA genes were not evident from the restriction map alone. The orientations of the $tRNA^{Tyr}$ genes on λHtM2 and λHtM4 were determined from the restriction endonuclease maps and defined by DNA sequencing. However, this approach for establishing the gene orientation did not work for the 6-2, 6-3 and 6-4 $tRNA^{Tyr}$ genes on λHtM6 because the nucleotide sequences of the regions between these $tRNA^{Tyr}$ genes were not known. The $tRNA^{Tyr}$ gene orientations of λHtM6 were determined by PCR using primer combinations that tested all possible orientations of the four $tRNA^{Tyr}$ genes. Only the primer combinations that reflected the gene orientations as they occurred on λHtM6 would yield PCR products that could be verified by hybridization with gene-specific probes. These PCR experiments not only revealed the tandem orientation of the $tRNA^{Tyr}$ genes on λHtM6, they also confirmed the restriction map which indicated the distances between these genes (Figure 18). The tRNA genes on λHtM4 are also arranged in tandem, but this arrangement is not unusual among the tRNA gene clusters characterized thus far (Chang *et al.* 1986; Doran *et al.*, 1987; Shortridge *et al.*, 1989).

The six human intron-containing $tRNA^{Tyr}$ gene sequences have high similarity to several other eukaryotic $tRNA^{Tyr}$ gene sequences, especially the *Xenopus laevis* tRNA gene used as a probe to screen the recombinant phage library. An alignment of $tRNA^{Tyr}$ gene sequences from a few eukaryotic organisms clearly illustrates the homology that exists amongst these genes (Figure 44). All of the eukaryotic $tRNA^{Tyr}$ genes which have been characterized thus far contain introns. In some cases (*Saccharomyces cerevisiae*, *Drosophila melanogaster*, and human) evidence has been presented to show that the intron is essential for the pseudouridine modification in the anticodon (Choffat *et al.*, 1988; Johnson and Abelson, 1983; van Tol and Beier, 1988).

The number of $tRNA^{Tyr}$ genes in the haploid human genome has been estimated at 12, with six intron-containing $tRNA^{Tyr}$ genes having been cloned thus far. This estimate is

Figure 44. Comparison of the isolated human $tRNA^{Tyr}$ gene sequences with $tRNA^{Tyr}$ gene sequences from the literature

The alignment was done on 16 $tRNA^{Tyr}$ gene-containing sequences. The character to show that a position in the alignment is perfectly conserved is '*'. The character to show that a position is well conserved is '·'. The tDNA sequences are shown in bold letters, while the intervening sequences are underlined. The sources and names of the sequences used for the alignment are listed below:

[1] *Xenopus*, TyrD (Gouilloud and Clarkson, 1986)

[2] *Xenopus*, TyrC (Gouilloud and Clarkson, 1986)

[3] *Drosophila*, Y85aa (Suter and Kubli, 1988)

[4] *Drosophila*, Y85ab (Suter and Kubli, 1988)

[5] *Drosophila*, Y85ad (Suter and Kubli, 1988)

[6] *Drosophila*, Y85ae (Suter and Kubli, 1988)

[7] *Drosophila*, Y85ac (Suter and Kubli, 1988)

[8] human, 4-1 (MacPherson, 1988)

[9] human, 6-1 (MacPherson and Roy, 1986)

[10] human, 6-3 (MacPherson, 1988)

[11] human, 6-2 (MacPherson and Roy, 1986)

[12] human, 6-4 (MacPherson, 1988)

[13] human, 4-2

[14] *Nicotiana rustica*, pNTT1 (Stange and Beier, 1986)

[15] *Arabidopsis thaliana*, pATT1 (Stange et al., 1988)

[16] *Arabidopsis thaliana*, pATT3 (Stange et al., 1988).

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          -60        -50        -40        -30        -20
           |          |          |          |          |
1     AAT--AGAAAC-----AAGAAGTTAACGCAATGA----------------C-CAA
2     -------GAAC-----AATTGAAGTCCACCAAGT----------------CACGG
3     T-CGCTCCTGA-----ACAAAA--------GGTG----------------AATTA
4     TGTGCGCATGC-----ATAAAA--------CGAA----------------CAACG
5     CTCGCA----C-----ATAAATGTACAAAACTTA----------------CATTG
6     AGTGCGAAAGC-----AAATGAATCCC--CCCAA----------------C-TAA
7     CG-----ATGA-----ATCGGATACCCGTTGTTT----------------AACCT
8     AGC--GC--TCCGGTTTT---------TCTGT--GCTGAACCTCAGGGGACGCCG
9     ---------TCCA-----------------TTTGCGGAAAGTCCAGTGAT-CCA
10    CTCAAGCATTCCAGGGATGTGGCTCCCTCTGTTTGCAGAAAGTCCAATGAA-CCA
11    TTC-------------------CTCC----ATTTGCAGAAAGTCCAGTGAC-CCA
12    GTCTTGCATT----------------------TGCTGAAAGATCAATGAC-CCA
13    A-------------TGTTATGGAGAC-----AAGGCGG---CACCCGGGA----A
14    TGGACGACTT-------------------------CTATATAGG-TATGAGATTA
15    TAGAATATTT---------------------AGATCAGTACA-C-ATGCAT---G
16    GCTTCTCTT----------------------GAATCA--TCAAC-ATGCATAAAG

          -20        -10         +1        +10        +20        +30
           |          |          |          |          |          |
1     GC-CTGCATGAACATC----CCTTCGATAGCTCAGCTGCTAGAGCGGAGGACTGT
2     GCGATGGGTGCCCCACCGG-CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGT
3     GC-C-GCAAGCGAATTTAA-CCTTCGATAGCTCAGTTGGTAGAGCGGTGGACTGT
4     GC-C-AAGTTTGAACTCAA-CCTTCGATAGCTCAGTTGGTAGAGCGGTGGACTGT
5     GC-C-ACCCGAGATTCAAA-CCTTCGATAGCTCAGTTGGTAGAGCGGTGGACTGT
6     GC-C-CCCCA-----CCAA-CCTTCGATAGCTCAGTTGGTAGAGCGGTGGACTGT
7     ACTC-CCCAGCACGCCC---CCTTCGATAGCTCAGTTGGTAGAGCGGTGGACTGT
8     ACAC-----ACGTACACGTCCCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGT
9     GCTCTTGCAGCGTGCAC---CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGT
10    GCTTTGATAGCATGCAT---CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGT
11    GCCTTAACAGTGTGCAT---CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGT
12    ACCCCAGAAACGTGCGC---CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGT
13    GCTGTGCCCGC-------TCCCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTGT
14    GAGTATCTCGCAAGA-----CCGACCTTAGCTCAGTTGGTAGAGCGGAGGACTGT
15    AAATAGAATACAAT------CCGACCTTAGCTCAGTTGGTAGAGCGGAGGACTGT
16    TGTTATAATACAAAA-----CCGACCTTAGCTCAGTTGGTAGAGCGGAGGACTGT
                          **..*..********.**********.*******
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                +40         +50         +60         +70         +80         +90
                 |           |           |           |           |           |
1       AGAGG--AATA-TAGCA------ATCCTTAGGTCGCTGGTTCAATTCCGGCTCGA
2       AGGTG--TGATCGAGCA------ATCCTTAGGTCGCTGGTTCGATTCCGGCTCGA
3       AGTTGGAAAAACATGCAATAGAAATCCATAGGTCGCTGGTTCAAATCCGGCTCGA
4       AGTTGGAAAATTATGCAATAGAAATCCATAGGTCGCTGGTTCAAATCCGGCTCGA
5       AGTTGGAAAA-CAAGCAATAGAAATCCATAGGTCGCTGGTTCAAATCCGGCTCGA
6       AGTTGGCAAA-CAAGCAATAGAAATCCATAGGTCGCTGGTTCAAATCCGGCTCGA
7       AGTTGG-AAAACATGCAATAGAAATCCATAGGTCGCTGGTTCAAATCCGGCTCGA
8       AG-CTACTTCCTCAGCAGGAGACATCCTTAGGTCGCTGGTTCGATTCCGGCTCGA
9       AGATTGTACAGACATTTGCGGACATCCTTAGGTCGCTGGTTCGATTCCGGCTCGA
10      AGATTGTATAGACATTTGCGGACATCCTTAGGTCGCTGGTTCGATTCCAGCTCGA
11      AGACTGCGGAAACGTTTGTGGACATCCTTAGGTCGCTGGTTCAATTCCGGCTCGA
12      AGCCTGTAGAAACATTTGTGGACATCCTTAGGTCGCTGGTTCGATTCCGGCTCGA
13      AGGCG-----CGCGCCCGTGGCCATCCTTAGGTCGCTGGTTCGATTCCGGCTCGA
14      AGTGGTA-----CTGCTGA-G--ATCCTTAGGTCACTGGTTCGAATCCGGTAGGT
15      AGTAG-A-----C-GCAGATT--ATCCTTAGGTCACTGGTTCGAATCCGGTAGGT
16      AGTTG-A-----C-GCAGATA--ATCCTTAGGTCACTGGTTCGAATCCGGTAGGT
        **  .         .  .     ****.******.*******.*.***.*...*.

                  +100        +110        +120        +130        +140
                    |           |           |           |           |
1       AGGAG------ATGACTTTTTTTTTTTTTTTATTCGTTCATTC---C------CC
2       AGGA---------CGCTTTGTTTTCCAATGCGAGCCTAAGAAGGAGA------TG
3       AGGATCG---AAAAGATATAAATACCTTTTTTTTATATATTTTGTA-GT-GCACA
4       AGGAT-------TTTTTGTAATATCCAATTCGTTTTTGTTTTTATA-GTAGCACC
5       AGGAATT---TTTTGTTATTTTTAATTTTTTTTTTTAAATTTAGTAA--TTACTTT
6       AGGATTT---TTTTTTCAGTTATATTTTTTTTATTGAATTTAGGCACGTTTTAAA
7       AGGATTT---TTTGTCGAATTTTTTACAAGCCATTTTTAATGCATTCTTG-----
8       AGGAGA---CAAGTGC----GGTTTTTTTTC--------TCCAG-------CTCC
9       AGGAAGTGCCCGATGCTTTTGCATGCAATGCCACCTGGTGCCTGGTC-AAACGCC
10      AGGAAGTGCGTGATGCTTTTGGTTAAAAGCCCTGCAGCTTCCAAGT---------
11      AGGAAGCGCCTGACTCTTTTGCGCACAATGC-------TGCCTGG----------
12      AGGAGCTGCCGTATTCTTTTGCACAC---GCACGCA----CCAAAAC-TA----C
13      AGGAGAGAC--------------------ACCCCCCCCCCCCCATTATTTTGTTGC
14      CGGA------TTTTG--TTTTTCTGTGTTCTTTGCTTTTT-CATTTCAT------
15      CGGA------ATTTGCTCCCACATGAGAGCTTTTTATTTTTC-TTTCGTT-----
16      CGGA------T----CATTAAAATTGAAAGTTTTTTTTTTCCATTTCGTTT----
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based on hybridizations of $tRNA^{Tyr}$ gene-specific oligonucleotide probes to placental DNA digested with restriction endonuclease *Eco*RI (van Tol and Beier, 1988). There are probably more than 12 $tRNA^{Tyr}$ genes per haploid human genome since it is possible for a single *Eco*RI restriction fragment to carry multiple genes, as is the case in λHtM4 and λHtM6. A 7.7 kb *Eco*RI restriction fragment from λHtM4 contains three tRNA genes, two $tRNA^{Tyr}$ genes and one $tRNA^{Ala}$ gene (Figure 16). A 9.5 kb *Eco*RI restriction fragment from λHtM6 also contains two $tRNA^{Tyr}$ genes (Figure 16). This suggests a minimum of 14 $tRNA^{Tyr}$ genes in the human genome. It is thus remarkable that all five of the $tRNA^{Tyr}$ genes characterized by PCR by Green *et al.* (1990) are species present on λHtM4 and λHtM6.

The nucleotide sequences of five of the six human tRNA genes studied were determined by MacPherson (1988) and the $tRNA^{Tyr}$ genes carried on plasmids pM6 and pM6128 have been published (MacPherson and Roy, 1986). Tyrosine tRNA genes identical to the ones carried on plasmids pJM4 and pJMS42 have been described by van Tol *et al.* (1987) and Green *et al.* (1990), respectively. The $tRNA^{Tyr}$ gene characterized by van Tol *et al.* (1987), named $tRNA_1^{Tyr}$, that is identical to the 4-1 $tRNA^{Tyr}$ gene, was isolated from a human-λ Charon 4A recombinant bacteriophage, λHtT1, on a 6.0 kb *Eco*RI fragment. These two $tRNA^{Tyr}$ genes share identical 5' and 3' flanking sequences (with the exception of a few polymorphisms) and could each be isolated on a 401 bp *Sma*I/*Hae*III DNA fragment. However, van Tol *et al.* (1987) did not detect the additional $tRNA^{Tyr}$ gene (4-2) that was 0.5 kb upstream of the first gene. Since the $tRNA_1^{Tyr}$ gene was found on a 6.0 kb *Eco*RI fragment and the 4-1 $tRNA^{Tyr}$ gene was found on a 7.7 kb *Eco*RI fragment, it is quite likely that the bacteriophage clone λHtT1 isolated by van Tol *et al.* (1987) overlaps with the bacteriophage clone λHtM4 isolated by MacPherson (1988). Evidence to support this claim of overlapping bacteriophage clones comes from a comparison of restriction endonuclease digests of λHtT1 and λHtM4 DNA with *Eco*RI. The restriction endonuclease digests of these two bacteriophage clones generate identical

patterns of DNA fragments, except for the $tRNA^{Tyr}$ gene-containing *Eco*RI DNA fragments. This situation would explain why a double digestion of λHtT1 DNA with restriction endonucleases *Sma*I and *Hae*III would fail to yield two DNA fragments that would hybridize with the $tRNA^{Tyr}$ gene-specific oligonucleotide probe used by van Tol *et al.* (1987).

The $tRNA^{Tyr}$ gene-containing plasmid subclones, which were partially sequenced by MacPherson (1988), were sequenced further and the nucleotide sequences of these clones were compared. The most striking feature of these nucleotide sequences is the high degree of homology present in the 5' flanking sequences of the four $tRNA^{Tyr}$ genes on λHtM6. While the high degree of homology in the 5' flanking sequences of 6-1 and 6-2 $tRNA^{Tyr}$ genes had been previously observed by MacPherson and Roy (1986), it is now evident that this homology is also shared with the 6-3 and 6-4 $tRNA^{Tyr}$ genes. The identity observed in the 5' flanking sequences of the four RNA^{Tyr} genes on λHtM6 ranged from 67 to 74% over 400 bp, when pairs of sequences were aligned. With more 5' flanking sequence available from the plasmid subclones pM6, pM612 and pM6IT, additional alignments were performed and similarities ranging from 58 to 75% were observed over 600 bp immediately upstream of the $tRNA^{Tyr}$ genes on these plasmids. These sequence analyses have shown that the 6-4 $tRNA^{Tyr}$ gene has the most divergent 5' flanking sequence of the four genes on λHtM6 (Figure 24). Similar analyses of the 3' flanking sequences of the $tRNA^{Tyr}$ genes revealed only limited regions of similarity ranging from 29 to 64% over 100 bp immediately downstream of five $tRNA^{Tyr}$ genes. The homologous regions in the 3' flanking sequences included the putative RNA polymerase III termination signals, consisting of at least four consecutive Ts. The 4-2 $tRNA^{Tyr}$ gene had the most divergent 3' flanking sequence of the six $tRNA^{Tyr}$ genes characterized.

The extensive homology in the 5' flanking sequences of the $tRNA^{Tyr}$ genes on λHtM6 makes this tRNA gene homocluster unusual. There are other examples of human tRNA gene clusters with homology in the 5' and 3' flanking sequences of isoaccepting

tRNAs. Ma *et al.* (1984) observed greater than 90% identity over 300 bp in both the 5' and 3' flanking sequences of two $tRNA^{Asn}$ genes. Homology has also been found in the flanking sequences of two $tRNA_i^{Met}$ genes (Santos and Zasloff, 1981), but the homology extends over only 110 bp of 5' and 70 bp of 3' flanking sequence. This high degree of homology suggests that the $tRNA^{Tyr}$ gene homocluster on λHtM6 has arisen by gene duplication events. Gene duplication may have occurred by either a series of reverse transcription events, followed by recombination events, or by unequal crossing over. Gene duplication by unequal crossing over is more likely, since it can account for both the formation of the $tRNA^{Tyr}$ gene homocluster and the high percentage of sequence similarity present in the flanking sequences.

In vitro transcription assays performed with the cloned tRNA genes have shown each of them to be transcriptionally active. The $tRNA^{Tyr}$ transcripts and cleavage products generated by the 293 cell extracts (Weil *et al.*, 1979) were similar in size to those observed by van Tol *et al.* (1987) using HeLa cell extracts. The preliminary *in vitro* transcription assays of the human $tRNA^{Tyr}$ genes reported by MacPherson and Roy (1986) found that pM6128 directed RNA synthesis at levels six-fold higher than pM6. When these experiments were repeated the transcription levels among $tRNA^{Tyr}$ genes carried on plasmids pM6128, pM6, pM612, pM6IT, and pJM4 were found to be nearly equal by visual examination of the autoradiographs. The conformation of the template DNA can affect transcription by RNA polymerase III, since supercoiled DNA molecules are much more transcriptionally active than relaxed DNA molecules (Sekiguchi *et al.*, 1989). However, the differences in expression first encountered between the $tRNA^{Tyr}$ genes probably arose from errors in DNA quantification. DNA quantification performed with a TKO 100 fluorimeter and Hoechst 33258 dye prevents RNA contamination from interfering with readings, since the dye is highly specific for double stranded DNA. This has ensured that equimolar amounts of template DNA were used in this study for each *in vitro* reaction.

Attempts to isolate expression-modulating extragenic sequence elements by expressing plasmid constructs that had flanking sequence deletions also failed to demonstrate any appreciable differences in expression levels. In fact, even the complete replacement of the native flanking sequences with vector sequence (i.e. the multiple cloning site of pBS) did not significantly alter expression of the $tRNA^{Tyr}$ genes. However, the DNA templates that carried $tRNA^{Tyr}$ genes lacking any native flanking sequences often directed the synthesis of much longer pre-$tRNA^{Tyr}$ transcripts. These longer transcripts were due to RNA polymerase III having to transcribe further before a stretch of four or more Ts was encountered, such sequences serve as RNA polymerase III transcription terminators (Bogenhagen and Brown, 1981). When pM6 #436 was used as the DNA template for *in vitro* transcription, the longer transcripts observed were due to the transcription of the two tandem 6-2 $tRNA^{Tyr}$ genes carried on this plasmid. While a slight decrease in the rate of expression was observed with these chimeric DNA templates, it was not enough of a difference to allow conclusions to be drawn with regard to the existence of upstream regulatory sequence elements. Since transcription factor IIIB (TFIIIB) interacts with sequences upstream of the mature coding sequence, near the transcription start site (Bartholomew *et al.*, 1991; Kassavetis *et al.*, 1991), the slight loss in $tRNA^{Tyr}$ transcriptional activity that occurred upon replacement of all the native flanking sequence with vector sequences is not surprising. At present only the 4-2 $tRNA^{Tyr}$ gene appears to be transcribed at lower levels than the others. However, this observation may be misleading because pJMS42 also carries a $tRNA^{Ala}$ gene that is transcriptionally active. The lower levels of transcription of the 4-2 $tRNA^{Tyr}$ gene may just reflect the competition between the two genes for transcription factors. The 4-2 $tRNA^{Tyr}$ gene will have to be transcribed alone before its transcriptional activity can be compared meaningfully to those of the other $tRNA^{Tyr}$ genes. Future experiments might also examine the ability of the other $tRNA^{Tyr}$ genes to compete with the 4-3 $tRNA^{Ala}$ gene for transcription factors.

The results of these *in vitro* transcription assays suggest that either there are no extragenic modulatory sequences present in the flanking sequences of these $tRNA^{Tyr}$ genes or that these sequence elements do not exert detectable effects under the *in vitro* conditions of these assays. The transcription factors that interact with these hypothetical modulating sequences may be inactive in the S-100 cell extracts because they are labile or expressed at low levels.

A putative Alu sequence was found in the 5' flank of the 6-2 $tRNA^{Tyr}$ gene. However, it does not appear to affect the transcriptional efficiency of the tRNA gene. Although the putative Alu element has 67% identity to a transcriptionally active Alu sequence described by Perlino *et al.* (1985), and has only one divergent position in its B box promoter element, it does not appear to be transcriptionally active *in vitro*. Alu sequences have been detected in or around other human tRNA gene clusters (Chang *et al.* 1986; Doran *et al.*, 1987; Shortridge *et al.*, 1989) and are not thought to influence tRNA gene transcription.

There have been several attempts to isolate extragenic regulatory sequences in the flanking sequences of human tRNA genes. While examples of 5' flanking sequences that modulate tRNA gene transcription have been described, a consensus sequence for an extragenic regulatory element has yet to be determined. It was observed by Shortridge *et al.* (1989) that deletions of 5' flanking sequence did not cause any significant change in transcriptional efficiency of a human $tRNA^{Thr}$ gene until deletions left only 2 bp of 5' flanking sequence upstream of the gene. However, the effects of the deletions became more apparent when the $tRNA^{Thr}$ gene had to compete with either a human $tRNA^{Pro}$ gene or a $tRNA^{Gly}$ pseudogene for transcription factors and RNA polymerase III. In competition experiments with other human tRNA genes, the plasmid constructs with less than 168 bp of 5' flanking sequence immediately upstream of the $tRNA^{Thr}$ gene were not able to compete for transcription factors as well as the plasmid constructs with additional 5' flanking sequence. Therefore, it was concluded by Shortridge *et al.* (1989) that the 168

bp of 5' flanking sequence immediately upstream of the $tRNA^{Thr}$ gene contains one or more cis-acting regulatory elements that are crucial for the high transcriptional activity of this gene. Gonos and Goddard (1990) studied the effects of 5' flanking sequence deletions on the *in vitro* transcription of a human $tRNA^{Glu}$ gene with HeLa cell extracts and found the efficiencies of the deletion clones correlated with their relative competitor strengths. However, to explain the transcriptional efficiencies of the deletion clones it was suggested that the 5' flanking sequence contained both a positive and a negative transcription modulator. When these experiments were repeated in a heterologous system (i.e. *Xenopus laevis* oocytes) the results were more straightforward, with increasing deletions of the 5' flanking sequence leading to decreasing transcription efficiency of the $tRNA^{Glu}$ gene. With data obtained from the expression of the human $tRNA^{Glu}$ gene in both homologous and heterologous systems, coupled to the new model for the assembly of transcription factors on genes transcribed by RNA polymerase III, Gonos and Goddard (1990) proposed a model for transcription modulation by extragenic sequences. Their model proposes that transcription modulation may occur by changes in TFIIIB activity via interaction with tissue-specific factors that recognize different upstream sequences.

Comparisons of sequences upstream of tRNA genes have identified short sequence elements that have been proposed to act as positive transcription modulators for these genes. Sajjadi and Spiegelman (1987) have proposed the sequence TNNCT as the general form of a positive transcription modulator for *Drosophila melanogaster* tRNA genes. There are examples of extragenic sequences which have been found upstream of the *Bombyx mori* $tRNA^{Ala}$ gene (Larson *et al.*, 1983), the human $tRNA^{Val}$ gene (Arnold *et al.*, 1987), the mouse $tRNA^{Asp}$ gene (Rooney and Harding, 1988), and the *Saccharomyces cerevisiae* $tRNA^{Leu}$ gene (Johnson and Raymond, 1984) that are known modulate transcription. Of the 23 *Drosophila melanogaster* tRNA gene sequences analyzed by Sajjadi and Spiegelman (1987) 13 genes had the TNNCT sequence in their 5' flanking sequence in the region from –25 to –45. However, the TNNCT sequence was

found upstream of 12 of the 14 tRNA genes that are transcribed at moderate to high efficiency. Similar sequence comparisons in *Saccharomyces cerevisiae* have also identified a canonical sequence, CAANAAA, as a positive transcription modulator in the upstream sequences of several tRNA and 5S RNA genes (Raymond and Johnson, 1984). A statistical analysis of the flanking regions of eukaryotic tRNA genes was performed to identify consensus sequences (Makalowski and Augustyniak, 1992). These conserved signals may play a role in transcription regulation, since sequences that are functionally more important evolve more slowly than less important ones. Makalowski and Augustyniak (1992) identified a conserved sequence between positions –32 and –27, $^{A}/_{T}$GAG, in the 5' flanking sequences of 18 of 50 tRNA genes analyzed from vertebrates. However, this consensus sequence was not found within 50 bp of any of the human tRNA genes characterized in this study. Those upstream elements that have been described are usually found up to 50 bp from the start of the mature coding sequence and tend to be AT-rich, however there has not yet been a report explaining how any of these sequence elements effect an increase in transcription.

The absence of any detectable regulatory elements in the 5' flanking sequences of the six $tRNA^{Tyr}$ genes and many other human tRNA genes suggests that these genes are under global regulation. The cellular concentration of tRNA can be controlled by regulating the availability of transcription factors and/or RNA polymerase III. Transcription-modulating extragenic sequences may only occur in the flanking sequences of tRNA genes whose expression must respond immediately to meet the needs of the cell. Since the six $tRNA^{Tyr}$ genes characterized in this study recognize the same codon in mRNA, the transcriptional efficiencies of these genes do not have to differ in response to codon preferences.

There is a growing body of evidence suggesting that TBP is required for the transcription of genes by RNA polymerase III. The uncertainty about the role of TBP in tRNA gene expression was caused by the presence of TBP in phosphocellulose fractions

containing TFIIIB and TFIIIC. This endogenous TBP masked the effect of added TBP on tRNA gene expression in earlier studies. In the 5' flanking sequences of the $tRNA^{Tyr}$ and $tRNA^{Ala}$ genes TATA-box elements have been found, except in the 5' flanking sequence of the 6-2 $tRNA^{Tyr}$ gene. However, only in the 5' flanking sequences of tRNA genes carried on pJMS42, 41 bp upstream of the 4-2 $tRNA^{Tyr}$ gene and 9 bp upstream of the 4-3 $tRNA^{Ala}$ gene, have classical TATA box elements been found within 200 bp of the tRNA gene. At present it is difficult to determine if these elements have an effect on transcription efficiencies. If purified TBP could be obtained readily, the expression of the $tRNA^{Tyr}$ and $tRNA^{Ala}$ gene constructs might be repeated with additional TBP supplementing the 293 cell extracts to determine if the native 5' flanking sequences can modulate transcription levels via interaction with TBP.

A report by van Tol *et al.* (1989) claimed that pre-$tRNA^{Tyr}$ transcripts are capable of autocatalytic intron excision, however no other published reports supporting their claim have appeared. Attempts at reproducing their results have been undertaken with pre-tRNA transcripts synthesized either by T7 RNA polymerase or 293 S-100 cell extracts. As shown in the Results, RNA self-cleavage was observed *in vitro* with pre-tRNA transcripts regardless of their origin, but, some pre-tRNA transcripts are more prone to cleavage than others. For example, 4-1 transcripts exhibited more RNA self-cleavage activity than all other pre-$tRNA^{Tyr}$ transcripts. For *in vitro* RNA cleavage to be detected the pre-$tRNA^{Tyr}$ transcripts had to be incubated for at least 2 hours at temperatures between 37 and 42°C. Although only a very small fraction of the pre-$tRNA^{Tyr}$ transcripts underwent self-cleavage, the amount of activity seen was comparable to other ribozyme reactions (Haseloff and Gerlach, 1988). While van Tol *et al.* (1989) provided evidence that the intron was excised, their observations and the RNA self-cleavages observed in this study can be explained simply as magnesium-promoted cleavage of pre-$tRNA^{Tyr}$ transcripts. While there are both specific and nonspecific tRNA cleavages promoted by metal ions, the specific cleavages involve the precise coordination of the metal ion with the

RNA. The best characterized example of a specific intramolecular metal-ion induced cleavage is the lead-promoted cleavage of yeast $tRNA^{Phe}$ between residues D_{17} and G_{18} (Werner *et al.*, 1976).

In order for these pre-$tRNA^{Tyr}$ transcripts to be catalytic they must adopt a specific tertiary conformation. Therefore, I hypothesized that anything which causes a significant perturbation of the pre-tRNA transcript's folding pattern should have an adverse effect on catalytic activity. This hypothesis was tested by performing self-cleavage experiments in the presence of specific oligonucleotides that were either identical or complementary in sequence to portions of the pre-tRNA transcripts to determine their effects on catalytic activity. It was observed that oligonucleotides complementary to the intron sequences were capable of inhibiting pre-tRNA self-cleavage. The degree of self-cleavage inhibition achieved by the oligonucleotide depended on its ability to anneal to the intron of the pre-tRNA transcripts; therefore, specific oligonucleotides could inhibit the cleavage of more than one species of pre-$tRNA^{Tyr}$. Oligonucleotides directed towards the exons had little if any affect on catalytic activity, except for an oligonucleotide complementary to the 5' half of $tRNA^{Tyr}$ (i.e. DSP 23). Instead of inhibiting self-cleavage, this oligonucleotide modified the cleavage pattern, which indicated a change in the reaction's specificity. This observation is intriguing because it has been observed that for tRNA splicing to occur the pre-tRNA transcript must adopt a conformation similar to the mature tRNA tertiary structure and the 3' splice site must be single-stranded (Lee and Knapp, 1985; Szekely *et al.*, 1988). It appears that the requirement for the single-stranded 3' splice site is important for intron excision, whether considering the activity of the tRNA splicing endoribonuclease or the suspected intrinsic catalytic activity of the pre-$tRNA^{Tyr}$ transcript. However, the pre-$tRNA^{Ala}$ transcripts, which lack an intervening sequence, also displayed some RNA cleavage *in vitro* when the incubations were performed at 46°C. The ability of both pre-$tRNA^{Tyr}$ and pre-$tRNA^{Ala}$ transcripts to undergo magnesium-promoted RNA self-cleavage suggests that tRNAs may share certain

tertiary features that permit metal-ion catalyzed cleavages. These two tRNA species are able to promote self-cleavage of specific phosphodiester bonds by coordinating magnesium ions. The catalytic activity of these tRNA species defines them as ribozymes, even though it is unlikely that these self-cleavage reactions occur *in vivo*. The first tRNA ribozyme characterized was yeast $tRNA^{Phe}$, which undergoes lead ion-promoted self-cleavage (Behlen *et al.*, 1990). But, Pb^{2+} is much less relevant in biological systems than is Mg^{2+}.

The results presented in this study are similar to those reported by van Tol *et al.* (1989), with respect to the numbers and sizes of the RNA self-cleavage products observed. The RNA self-cleavage activity cannot be described as intron excision until the cleavage sites on the pre-$tRNA^{Tyr}$ transcript are mapped or the RNA fragments are sequenced. The pre-$tRNA^{Tyr}$ cleavage products are similar in size to the RNA processing intermediates generated during *in vitro* transcription assays, which range in size from 50 to 70 nt. However, a more detailed comparison between the cleavage products and the RNA processing intermediates will be technically challenging because it will involve sequencing extremely small quantities of RNA.

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6. Appendix

Examples of the modulatory effects observed in the extragenic regions of eukaryotic tRNA genes

Saccharomyces

GENE(S)	COMMENTS	REFERENCE(S)
$tRNA_3^{Leu}$	Deletion experiments have shown that replacement of yeast DNA up to position -2 (relative to the mature coding sequence) leaves the $tRNA_3^{Leu}$ nearly transcriptionally inert in yeast extracts. There is a conserved sequence -15(TTTCAACAAATAAGT)-1 in the 5' flanking sequence of this gene, which is also found in the flanking sequence of many known yeast tRNA and 5S RNA genes. By examining the flanking sequence of the genes that contain this conserved sequence a canonical sequence (CAANAAA) has been determined. This conserved sequence has been shown to act as a positive modulator of transcription both *in vitro* and *in vivo*. This sequence element may represent a mechanism by which the transcription of specific tRNA genes can be enhanced to meet the demands caused by codon usage preferences.	Raymond and Johnson (1983) Johnson and Raymond (1984) Raymond *et al.* (1985)

Saccharomyces

GENE(S)	COMMENTS	REFERENCE(S)
$tRNA^{Tyr}$ (SUP4-o)	The expression of the ochre-suppressing $tRNA^{Tyr}$ gene was monitored *in vivo* by assaying the extent to which deletion mutants of this gene were able to suppress seven ochre mutations in *S. cerevisiae*. Phenotypic expression of SUP4 gene constructs is impaired when deletions come within 36 bp of the tRNA coding region and is further reduced as deletions near the coding region. Based upon the diversity of tRNA gene 5' flanking sequences and the difficulty obtaining point mutations which reduce expression, it is doubtful that the 5' flanking sequences have any critical sequence-specific contacts with the transcription complex. It has also been observed that deletions in the 3' flanking sequences, which leave 5 or fewer consecutive T residues, significantly reduce the *in vitro* and *in vivo* expression of the SUP4-o gene. These 3' flanking sequence deletions also have a reduced ability to compete for transcription factors, suggesting that these downstream sequences play a role in binding these factors.	Shaw and Olson (1984) Allison and Hall (1985)

Caenorhabditis

GENE(S)	COMMENTS	REFERENCE(S)
$tRNA_i^{Met}$	All five of the $tRNA_i^{Met}$ genes are transcribed by homologous cell extracts but there are notable differences between these genes in expression. The $tRNA_i^{Met}$ genes Cetmet 3 and Cetmet 5 are more efficient transcription templates than Cetmet 1, Cetmet 2 and Cetmet 4; however, since the coding regions are all identical the flanking sequences must be modulating the transcription of these genes.	Honda *et al.* (1986) Khosla and Honda (1989)

Bombyx

GENE(S)	COMMENTS	REFERENCE(S)
$tRNA_C^{Ala}$	The promoter region of the $tRNA_C^{Ala}$ gene occupies a region of ~160 bp, that includes the coding region plus at least 13 bp upstream of the transcription start site	Sprague *et al.* (1980)
$tRNA_{SG}^{Ala}$	and at least 48 bp downstream of the termination site. A group of three sequences has been found at nearly identical positions upstream of several *Bombyx mori* tRNA and 5S RNA genes. Experiments have shown the sequence, -34(GACTTTATATTAGTAATTTTTGCA)-11, to be essential for transcriptional activity. Since the $tRNA_{SG}^{Ala}$ (silkgland-specific) gene lacks a significant portion of this sequence, this region may play a regulatory role in tissue-specific control of $tRNA^{Ala}$ gene transcription. In this system a domain which includes the coding and 3' flanking sequences is responsible for the binding of known transcription factors, while the role of the upstream sequence is still not understood.	Larson *et al.* (1983) Wilson *et al.* (1985) Young *et al.* (1986) Young *et al.* (1991b)

Drosophila

GENE(S)	COMMENTS	REFERENCE(S)
$tRNA^{Arg}$	By studying the expression of 5' and 3' deletion mutants of the $tRNA^{Arg}$ gene it was observed that removal of any *Drosophila* sequence between -10 and +85 reduces the ability of the template to compete for transcription factors. Optimal transcription factor binding is dependent on the sequences extending from the 5' end of the transcription start site to more than 10 bp downstream from the transcription termination sequence. Expression of this *Drosophila* gene in *S. cerevisiae* extracts shows deletions to between -21 and -17 (relative to position +1 of the mature coding sequence) drastically reduces transcription, while *in vivo* expression of $tRNA^{Arg}$ in *S. cerevisiae* is curtailed only by deletions to between -17 and -11.	Schaack *et al.* (1983) Sharp *et al.* (1983) Schaack and Söll (1985)
$tRNA_5^{Asn}$	Three identical $tRNA_5^{Asn}$ genes have been cloned which have different 5' and 3' flanking sequences and have different transcription efficiencies. The differences in transcription efficiency were attributable to the 5' flanking sequences, with the results from deletion analyses defining an upstream control region to between positions -33 and -20. If the upstream element contributes to the nucleation of strand separation by RNA polymerase III, this could explain the lack of sequence motifs in this regulatory element. The modulatory effects observed for tDNA transcription could then be explained by RNA polymerase III interacting unequally with different sequences in this upstream element.	Lofquist and Sharp (19860 Lofquist *et al.* (1988)

Drosophila

GENE(S)	COMMENTS	REFERENCE(S)
$tRNA^{His}$	A *Drosophila* $tRNA^{His}$ gene is transcribed efficiently while a $tRNA^{His}$ pseudogene is not. Deletion analysis of the bona fide gene revealed that the presence of the wild-type 5' flanking sequence is important for factor binding to the internal control regions and for stable complex formation. Both these genes are poorly transcribed in HeLa cell extracts regardless of the 5' flanking sequence, but they do compete for HeLa transcription factors.	Cooley *et al.* (1984)
$tRNA_2^{Lys}$	Deletion analysis of a *Drosophila* $tRNA_2^{Lys}$ gene has revealed that a sequence (GGCAGTTTTTG) located 13 nucleotides upstream from the mature coding sequence that is responsible for transcriptional repression. This sequence is found in all known *Drosophila* $tRNA_2^{Lys}$ genes; however, its ability to repress transcription depends upon its positioning in the 5' flanking sequence relative to the tRNA coding region.	DeFranco *et al.* (1981)
$tRNA_4^{Val}$	It has been demonstrated that the deletion of a five base sequence, TCGCT, between nucleotides -34 and -38 (relative to the mature coding sequence) reduces transcription by 90%. By creating a number of site-specific changes in the TCGCT sequence, the results indicate that a general form of the sequence TNNCT is a positive modulator for the transcription of *Drosophila* tRNA genes.	Rajput *et al.* (1982) Sajjadi and Spiegelman (1987)

Xenopus

GENE(S)	COMMENTS	REFERENCE(S)
$tRNA_i^{Met}$	Inhibitory sequences were found in the 5' flanking sequences of $tRNA_i^{Met}$ genes. The most inhibitory one, -20(TGCGCGTGC)-12, consists of 9 bp of alternating purines and pyrimidines, while the weaker inhibitory sequence, -43(ATGCACAGCGCA)-32, is composed of 12 bp of alternating purines and pyrimidines with one residue out of alternation.	Hipskind and Clarkson (1983)
$tRNA^{Tyr}$	A solitary $tRNA^{Tyr}$ gene is transcribed at levels ~6-fold greater than a $tRNA^{Tyr}$ gene from a gene cluster. The two genes differ by only one purine transition within the coding region; however, there are extensive differences within the 5' and 3' flanking sequences. The 12 bp immediately upstream of the dispersed gene are sufficient for efficient transcription *in vitro*; however, there is also an effect from sequences even further upstream of the initiation site on differential expression. Further analyses have identified four $tRNA^{Tyr}$ genes, two oocyte-type and two somatic-type, by their different 5' leader and intervening sequences present in the unspliced pre-tRNA transcripts. The expression of these genes appears to be developmentally regulated with the switch from oocyte-type to somatic-type occurring during embryogenesis.	Gouilloud and Clarkson (1986) Stutz *et al.* (1989)

MOUSE

GENE(S)	COMMENTS	REFERENCE(S)
$tRNA_1^{Asp}$ $tRNA_2^{Asp}$	The $tRNA_2^{Asp}$ gene has 5-fold greater transcriptional activity and a greater ability to form stable complexes with transcription factors than the $tRNA_1^{Asp}$ gene. The differential expression of these genes results from two sequences: a stimulatory sequence, -53(CGGTCTTGAATATCTATTCAAGA)-31, that increases transcriptional efficiency and a sequence, -9 to-1, that enhances transcription factor binding. Thus, eukaryotic tRNA gene transcription can be modulated by separate and distinct 5' flanking sequences	Rooney and Harding (1988)
$tRNA^{His}$	Four identical $tRNA^{His}$ genes contain various amounts of short, conserved 5' and 3' flanking sequences adjacent to the coding regions. Deletion analysis of one of these genes determined that deletions which removed sequences from positions -9 to -3 reduce transcriptional activity 5-fold. The construct with only 3 bp of 5' flanking sequence also had a reduced ability to compete with other tRNA genes for *in vitro* expression.	Morry and Harding (1986)

MOUSE

GENE(S)	COMMENTS	REFERENCE(S)
$tRNA_i^{Met}$	The coding region of a mouse $tRNA_i^{Met}$ gene is identical to a human $tRNA_i^{Met}$ gene. Another striking similarity between these genes is the homology in the 5' flanking sequences. Stretches of sequences 6 to 32 bp in length that are 76-100% identical, which are separated by short stretches of unrelated sequences, have been observed in the 5' flanks of these two genes. This is one of the first examples of sequence homology observed in the 5' flanking sequences of tRNA genes from distantly related mammals, which suggests that these conserved upstream sequences are required for some aspect of $tRNA_i^{Met}$ gene function.	Han *et al.* (1984)

HUMAN

GENE(S)	COMMENTS	REFERENCE(S)
$tRNA^{Val}_{IAC}$ (major) $tRNA^{Val}_{CAC}$ (minor)	The difference in expression between the major and minor $tRNA^{Val}$ species is 10-fold. Both the 5' and 3' flanking sequences of the major $tRNA^{Val}$ gene promote increased transcription, but only the 5' leader sequence has a positive influence on stable preinitiation complex formation. Transcription experiments have demonstrated that an extragenic region, between -51(GAATTCAGGACTAGTCTTTTAGGTCAAAAAGAAGAA)-16, acts as an expression modulator by facilitating factor binding. The lack of homology seen in the flanking sequences suggests that ECRs of human tRNA genes consist of fairly individual DNA elements that share little, if any, sequence homology.	Arnold *et al.* (1986) Arnold and Gross (1987) Arnold *et al.* (1988)